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(54) Title: MODULATION OF TOLERANCE BY ALTERING NFAT SIGNALLING

(57) Abstract: Methods and composition for modulating tolerance by altering the interaction of the T cell receptor-activated transcription factor NFAT with an NFAT-ligand, e.g., a CD28-activated transcription factor, such as AP-1 (e.g., Fos/Jun, Jun/Jun dimers) and NFκB/Rel, are described.

MODULATION OF TOLERANCE BY ALTERING NFAT SIGNALLING

Related Applications

This application claims priority to U.S. provisional application number 60/178,399 filed on January 27, 2000, the contents of which are incorporated herein by reference.

Background of the Invention

The diverse antigen receptors expressed on immune cells recognize not only foreign antigens expressed on pathogenic cells and organisms, but also self antigens expressed on the body's own tissues and the nonpathogenic antigens that elicit allergic reactions. A major clinical objective in transplant medicine and the treatment of autoimmune and allergic disease is the induction of tolerance to allo-, self, and environmental antigens, respectively. There is also much evidence for immune tolerance induced by tumors, leading to functional inactivation of T cells and a resulting inability to mount a tumor-specific response. While tolerance to self antigens is imposed, in large part, during development, through the process of clonal elimination, there is also a clear component of non-deletional tolerance in the periphery. The basic mechanisms underlying peripheral tolerance remain obscure.

Costimulation is necessary for a productive response to antigen (reviewed in Jenkins M.K. (1994) *Immunity* 1: 443-446; Lenschow D.J. et al. (1996) *Annu Rev Immunol* 14: 233-258; Parijs L.V. et al. (1996) *Science* 280: 243-248.). In T cells, a predominant costimulatory receptor is CD28, which binds the costimulatory ligands B7-1 (CD80) and B7-2 (CD86) expressed on the surface of the antigen-presenting cells (APC). Combined engagement of TCR and CD28 results in full activation of a number of signaling pathways that ultimately lead to to IL-2 production and cell proliferation. TCR engagement in the absence of costimulation results in a partial response. The incompletely stimulated T cells enter a long-lived unresponsive state, known as tolerance or anergy. Anergic T cells proliferate weakly and make little IL-2 in response to antigen. These cells become incapable of responding to complete stimulation through both TCR and CD28 (reviewed in Schwartz R.H. (1990) *Science*

248: 1349-1356; and Schwartz R.H. (1996) *J Exp Med.* 184(1):1-8).

Antigen binding to the B cell antigen receptor causes analogous biochemical and biological effects to antigen binding to the T cell receptor. B cell receptor ligation results in B cell proliferation and induces the expression of T cell costimulatory molecules such as B7-2, priming the B cell to produce antibodies. Like CD28 on T cells, activation of CD19 costimulatory receptor complex on B cells synergistically augments B cell activation and antibody production (Dempsey, P.W. et al. (1996) *Science* 271:348-350). B cell receptor activation in the absence of CD19 costimulation results in a partial, tolerant or anergic response. Moreover, just as inappropriate expression of B7 can provoke T cell autoimmunity, disregulated expression of CD19 can provoke spontaneous autoantibody production by B cells that would otherwise remain tolerant (Inaoki et al. (1997) *J. Exp. Med.* 186:1923-1931).

Summary of the Invention

The present invention is based, in part, on the discovery that imbalanced activation of the T cell receptor-activated transcription factor NFAT, relative to the activation of other transcription factors also induced during the complete immune response, e.g., a CD28-activated transcription factor, such as AP-1 (e.g., Fos/Jun, Jun/Jun dimers) and NFκB/Rel, promotes or induces anergy or tolerance. The complete set of these transcription factors that are turned on during a productive immune response may hereafter be referred to as "productive transcription factors". Because these transcription factors may also interact physically (e.g. AP-1) or functionally (e.g. NFκB/Rel) with NFAT, they may sometimes be referred to hereafter as "NFAT ligands". It is believed that imbalanced NFAT activation turns on a distinct genetic program associated with the anergic or tolerant state. Among the nucleic acids turned on under these conditions (also referred to herein as "anergy-associated nucleic acids") there are some whose products have a negative feedback effect on the production of an immune response, e.g., these nucleic acid products may uncouple an antigen receptor from the proximal signaling pathways.

Accordingly, in general, the invention features, a method of modulating an NFAT-mediated immune response, e.g., modulating tolerance, in a subject. The method includes:

administering to a subject an agent, which modulates (e.g., inhibits or activates) one or more NFAT signaling activities (e.g., modulation of NFAT-NFAT ligand signaling), in an amount sufficient to modulate (e.g., inhibit or activate) an NFAT-mediated immune response.

In a preferred embodiment, the agent modulates (e.g., inhibits or activates) one or more of the following NFAT signaling activities: (i) an interaction, e.g., binding or a functional interaction, between NFAT and an NFAT ligand (e.g., a transcription factor, such as AP-1, NFκB/Rel); (ii) the level of an NFAT-NFAT ligand complex; (iii) the activity or expression of NFAT and/or an NFAT ligand; (iv) the activity and/or expression of at least one other component of NFAT signaling, e.g., an upstream component of NFAT or NFAT-ligand signaling (e.g., a protein kinase or phosphatase that modulates NFAT or NFAT-ligand expression or activity, e.g., calcineurin), or a downstream component of NFAT or NFAT-ligand signaling, e.g., an agent which modulates the activity or expression of one or more anergy-associated nucleic acids or proteins, e.g., a protein involved in a proteolytic program (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1)); or (v) the activity and/or expression of at least one other component of NFAT ligand signaling, e.g., an upstream component of NFAT ligand signaling (e.g., a protein kinase or phosphatase that modulates NFAT ligand expression or activity, e.g., protein kinase C (PKC), (e.g., protein kinase C theta), MAP kinase, Ras, and Raf), or a downstream component of NFAT-NFAT ligand signaling.

In a preferred embodiment, the agent increases, e.g., selectively increases, one or more NFAT signaling activities, and/or inhibits or reduces one or more NFAT-NFAT ligand signaling activities. In one embodiment, the agent increases NFAT expression by e.g., upregulating transcription of an NFAT nucleic acid, or increasing the stability of an NFAT mRNA. In other embodiments, the agent may increase the level of free or uncomplexed NFAT in a cell, e.g., reduces the level of an NFAT-

NFAT ligand (e.g., an NFAT-AP1) complex, by e.g., increasing the expression or activity of NFAT, or decreasing the expression or activity of an NFAT ligand, e.g., AP-1. In one embodiment, the agent increases NFAT activity by, e.g., competitively or noncompetitively, inhibiting or reducing an NFAT-NFAT ligand interaction. In other embodiments, the agent modifies (directly or indirectly) NFAT, or an NFAT ligand, such that an interaction between NFAT and its ligand is reduced or inhibited. Such modification may result from, e.g., an alteration in an NFAT or NFAT ligand phosphorylation state, calcium binding properties, among others. In other embodiments, the agent increases the activity or expression of calcineurin, by, e.g., increasing intracellular calcium concentration. In yet other embodiments, the agent inhibits or reduces the activity or expression of an NFAT ligand activator chosen from protein kinase C (PKC), e.g., protein kinase C theta, MAP kinase, Ras, and Raf. In yet another embodiment, the agent increases the expression or activity of one or more anergy-associated nucleic acids or proteins, e.g., a protein involved in a proteolytic program (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1)).

Preferably, the increased NFAT signaling induces, or promotes anergy or tolerance, in a subject, thereby inhibiting, or reducing, an unwanted or detrimental immune response in the subject.

In a preferred embodiment, the subject is a human, e.g., a patient suffering from an unwanted immune response, e.g., an autoimmune disorder (including, for example, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, diabetes, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), allergy (e.g., atopic allergy), asthma (e.g., extrinsic or intrinsic asthma), a reaction to a xeno- or allo-antigen, graft-vs.-host disease, and transplant rejection.

In a preferred embodiment, the agent decreases, e.g., selectively decreases, one or more NFAT signaling activities, and/or enhances one or more NFAT-NFAT ligand signaling activities. In one embodiment, the agent may decrease NFAT expression by, e.g., downregulating transcription of an NFAT nucleic acid, or decreasing the stability of an NFAT mRNA. In other embodiments, the agent may increase the level of complexed NFAT in a cell, e.g., increase the level of an NFAT-NFAT ligand (e.g., an NFAT-AP1) complex, by e.g., increasing the expression or activity of NFAT and an NFAT ligand, e.g., AP-1. In one embodiment, the agent increases NFAT-NFAT ligand activity by, e.g., increasing an NFAT-NFAT ligand interaction. In yet another embodiment, the agent activates the activity or expression of a downstream component of NFAT-NFAT ligand signaling. In other embodiments, the agent modifies (directly or indirectly) NFAT or an NFAT ligand, such that an interaction between NFAT and its ligand is increased. Such modification may result from, e.g., an alteration in an NFAT or NFAT ligand phosphorylation state, calcium binding properties, among others. In other embodiments, the agent increases the activity or expression of an NFAT activator, e.g., calcineurin, or an NFAT ligand activator chosen from protein kinase C (PKC), e.g., protein kinase C theta, MAP kinase, Ras, and Raf. In yet another embodiment, the agent decreases the expression or activity of one or more anergy-associated nucleic acids or proteins, e.g., a protein involved in a proteolytic program (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1)).

Preferably, the increased NFAT-NFAT ligand signaling blocks or reduces tolerance, e.g., ongoing tolerance, or the initiation of tolerance, in a subject, thereby enhancing the subject's immunity. For example, an increase in one or more NFAT-NFAT ligand activities may be useful in treating or preventing, in a subject, a cancer (e.g., a tumor, a soft tissue tumor, or a metastatic lesion), or a pathogenic infection, e.g., a viral, bacterial, or parasitic infection.

In a preferred embodiment, the subject is a human, e.g., a cancer patient, or a subject in need of heightened immune surveillance, e.g., a patient suffering from a other or a subject suffering from a pathogenic infection, e.g., a viral (e.g., HIV), bacterial, or parasitic infection.

In those embodiments where tolerance is induced or promoted, the agent is an inhibitor of NFAT-NFAT ligand signaling. Such agent can be one or more of: a small molecule (e.g., a chemical agent having a molecular weight of less than 2500 Da, preferably, less than 1500 Da), a chemical, e.g., a small organic molecule, e.g., a product of a combinatorial library; a polypeptide (e.g., an antibody such as an intrabody), a peptide, a peptide fragment, a peptidomimetic, an antisense, a ribozyme, which inhibits NFAT or NFAT ligand expression or activity. In a preferred embodiment, the agent blocks or reduces an interaction between NFAT and an NFAT-ligand, e.g., AP-1, for example, by binding to the interactive site in NFAT or an NFAT ligand, e.g., AP-1. The agent can be a soluble fragment of NFAT; an NFAT peptide or a modified form thereof, e.g., a VIVIT peptide; a soluble fragment of an NFAT ligand, for example, a soluble fragment of an AP-1 component (e.g., a soluble fragment of Fos or Jun); a small molecule; a peptide or a polypeptide, e.g., a product of a peptide combinatorial library; a protein or peptide selected in a phage display or other multiple binding-based assay for its ability to inhibit an NFAT-NFAT ligand interaction; a peptidomometic, e.g., a VIVIT peptidomimetic.

In those embodiments where tolerance is induced or promoted, the agent is an inhibitor of an upstream activator of an NFAT ligand. In one embodiment, the NFAT ligand inhibitor inhibits or reduces the activity or expression of an NFAT ligand activator, e.g., an activator chosen from protein kinase C (PKC), e.g., protein kinase C theta, MAP kinase, Ras, and Raf. Such agent can be one or more of: a small molecule (e.g., a chemical agent having a molecular weight of less than 2500 Da, preferably, less than 1500 Da), a polypeptide (e.g., an antibody, such as an intrabody), a peptide, a peptide fragment, a peptidomimetic, an antisense, a ribozyme, which inhibits the expression or activity of the upstream activator of the NFAT ligand. .

In those embodiments where tolerance is induced or promoted, the agent is an activator (e.g., increases the activity or the expression) of at least one anergy-associated nucleic acid or protein. Exemplary anergy-associated nucleic acids or

proteins include e.g., a protein involved in a proteolytic program (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1)). In those embodiments where tolerance is inhibited or reduced, the agent is an activator of an upstream activator of an NFAT ligand. In one embodiment, the NFAT ligand activator enhances the activity or expression of an NFAT ligand activator, e.g., an activator chosen from protein kinase C (PKC), e.g., protein kinase C theta, MAP kinase, Ras, and Raf. Such agent can be one or more of: a small molecule (e.g., a chemical agent having a molecular weight of less than 2500 Da, preferably, less than 1500 Da), a polypeptide (e.g., an antibody, such as an intrabody), a peptide, a peptide fragment, a peptidomimetic, an antisense, a ribozyme, which inhibits the expression or activity of the upstream activator of the NFAT ligand. .

In those embodiments where tolerance is inhibited or reduced, the agent is an inhibitor (e.g., increases the activity or the expression) of at least one anergy-associated nucleic acid or protein. Exemplary anergy-associated nucleic acids or proteins include e.g., a protein involved in a proteolytic program (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1)). In preferred embodiments, the NFAT protein is selected from the group consisting of: NFAT1, NFAT2, NFAT3, and NFAT4.

In a preferred embodiment, the NFAT-NFAT ligand (e.g., AP-1) interaction is modulated, e.g., inhibited or activated: prior to exposure to an antigen; during exposure to an antigen; after exposure to an antigen; or a combination thereof.

The agents described herein can be administered by themselves, or in combination with at least one more agent. In one embodiment, a combination of the agents described herein, e.g., a modulator of an anergy-associated nucleic acid can be administered in combination with an inhibitor of an NFAT-NFAT ligand interaction, and /or a modulator of an upstream activator of an NFAT ligand. In other embodiments, a modulator of a costimulatory pathway can be coadminstered. These modulators can be administered prior to, simultaneously with, or after the administration of one or more of the agents described herein. For example, a modulator of a costimulatory receptor or its ligands (e.g., CD28/B7 or CD19/ligand) can be administered in combination with one or more of the agents described herein. In those embodiments where tolerance is increased, an inhibitor of a costimulatory pathway (e.g., at least one blocker, e.g., an inhibitor of the CD40 ligand-CD40 interaction (e.g., an anti-CD40L antibody), an inhibitor of the CD28-B7, or the CTLA4-B7 interaction (e.g., a soluble CTLA4, e.g., a CTLA4 fusion protein, e.g., a CTLA4 immunoglobulin fusion, e.g., CTLA4/Ig), or any combination thereof) can be coadministered.

The agents described herein may also be administered in combination therapy, i.e., combined with other agents, e.g., therapeutic agents, that are useful for treating disorders, such as cancers, immune cell- mediated disorders, or infections.

In another aspect, the invention features, a method for increasing an NFAT-mediated immune response, e.g., inducing or promoting, anergy in a cell, or tolerance in a subject. The method includes:

contacting a cell (e.g., an immune cell), or administering to a subject, an agent, e.g., an agent as described herein, which activates one or more NFAT signaling activities (e.g., inhibits of NFAT-NFAT ligand signaling), in an amount sufficient to activate an NFAT-mediated immune response.

In a preferred embodiment, the agent modulates one or more of the following NFAT signaling activities: (i) inhibits an interaction, e.g., binding, between NFAT and an NFAT ligand (e.g., a transcription factor, such as AP-1, NFκB/Rel); (ii) decreases the level of an NFAT/NFAT ligand complex; (iii) increases the activity or expression of NFAT, (iv) decreases the activity or expression of an NFAT ligand; (iv)

increases the activity and/or expression of at least one other component of NFAT signaling, e.g., an upstream component of NFAT signaling (e.g., a protein kinase or phosphatase that modulates NFAT expression or activity, e.g., calcineurin), or a downstream component of NFAT signaling, e.g., an agent which modulates the activity or expression of one or more anergy-associated nucleic acids or proteins, e.g., a protein involved in a proteolytic program (e.g., a protease, such as a RING finger protein) or a cytokine, e.g., IL-2, IL-13, IFN-γ, IL-10, TNF-α, and MIP-1α; or (v) reduces the activity and/or expression of at least one other component of NFAT ligand signaling, e.g., an upstream component of NFAT ligand signaling (e.g., a protein kinase or phosphatase that modulates NFAT ligand expression or activity, e.g., protein kinase C (PKC), (e.g., protein kinase C theta), MAP kinase, Ras, and Raf), or a downstream component of NFAT-NFAT ligand signaling.

In a preferred embodiment, the cell is an immune cell, e.g., a T or a B cell.

In a preferred embodiment, the subject is a human, e.g., a patient suffering from an unwanted immune response, e.g., an autoimmune disorder (including, for example, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, diabetes, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), allergy (e.g., atopic allergy), asthma (e.g., extrinsic or intrinsic asthma), a reaction to a xeno- or allo-antigen, graft-vs.-host disease, and transplant rejection.

In another aspect, the invention features, a method for decreasing an NFAT-mediated immune response, e.g., reducing or inhibiting, anergy in a cell, or tolerance in a subject. The method includes:

contacting a cell (e.g., an immune cell), or administering to a subject, an agent, e.g., an agent as described herein, which inhibits one or more NFAT signaling activities (e.g., activates of NFAT-NFAT ligand signaling), in an amount sufficient to inhibit an NFAT-mediated immune response.

In a preferred embodiment, the agent modulates one or more of the following NFAT signaling activities: (i) activates an interaction, e.g., binding, between NFAT and an NFAT ligand (e.g., a transcription factor, such as AP-1, NFκB/Rel); (ii)

increases the level of an NFAT/NFAT ligand complex; (iii) increases the activity or expression of NFAT, (iv) increases the activity or expression of an NFAT ligand; (iv) increases the activity and/or expression of at least one other component of NFAT signaling, e.g., an upstream component of NFAT signaling (e.g., a protein kinase or phosphatase that modulates NFAT expression or activity, e.g., calcineurin), or a downstream component of NFAT signaling, e.g., an agent which modulates the activity or expression of one or more anergy-associated nucleic acids or proteins, e.g., a protein involved in a proteolytic program (e.g., a protease, such as a RING finger protein) or a cytokine, e.g., IL-2, IL-13, IFN-γ, IL-10, TNF-α, and MIP-1α; or (v) increases the activity and/or expression of at least one other component of NFAT ligand signaling, e.g., an upstream component of NFAT ligand signaling (e.g., a protein kinase or phosphatase that modulates NFAT ligand expression or activity, e.g., protein kinase C (PKC), (e.g., protein kinase C theta), MAP kinase, Ras, and Raf), or a downstream component of NFAT-NFAT ligand signaling. .

In a preferred embodiment, the cell is an immune cell, e.g., a T or a B cell.

In a preferred embodiment, the subject is a human, e.g., a cancer patient, or a subject in need of heightened immune surveillance, e.g., a patient suffering from a other or a subject suffering from a pathogenic infection, e.g., a viral (e.g., HIV), bacterial, or parasitic infection.

In another aspect, the invention features, a method for modulating, e.g., inhibiting, a protein-protein interaction, e.g., binding, between NFAT and an NFAT ligand (e.g., AP-1). The method includes: contacting the NFAT ligand (e.g., AP-1), or NFAT with an agent that modulates, e.g., inhibits, an interaction between NFAT ligand (e.g., AP-1) and NFAT, e.g., an agent as described herein, such that the protein-protein interaction between the NFAT ligand (e.g., AP-1) and the NFAT is modulated, e.g., inhibited.

The subject method can be used on cell-free conditions (e.g., a reconstituted system), on cells in culture, e.g. *in vitro* or *ex vivo* (e.g., cultures comprising immune cells, e.g., T cells). For example, cells can be cultured *in vitro* in culture medium and a peptide, or a nucleic acid, as described herein, can be introduced to the culture

medium. Alternatively, the method can be performed on cells present in a subject, e.g., as part of an *in vivo* (e.g., therapeutic or prophylactic) therapy protocol.

In a preferred embodiment, the level of protein-protein interaction is detected by the formation of an NFAT-NFAT ligand complex.

In a preferred embodiment, the NFAT-NFAT ligand interaction is inhibited. Preferably, such inhibition results in increased NFAT signaling, e.g., the increased expression of one or more anergy-associated nucleic acids, e.g., an anergy-associated nucleic acid as described herein; the induction of tolerance; and/or the inhibition or reduction of an unwanted or detrimental immune response in a subject.

In a preferred embodiment, the subject is a human, e.g., a patient suffering from an unwanted immune response, e.g., an autoimmune disorder (including, for example, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, diabetes, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), allergy (e.g., atopic allergy), asthma (e.g., extrinsic or intrinsic asthma), a reaction to a xeno- or allo-antigen, graft-vs.-host disease, and transplant rejection.

In another aspect, the invention features, a method of modulating (e.g., increasing or decreasing) the expression of one or more anergy-associated nucleic acids. The method includes:

contacting a cell (e.g., a T or a B cell) with an agent, e.g., an agent as described herein, that modulates NFAT signaling and/or NFAT-NFAT ligand signaling;

allowing expression of the anergy-associated nucleic acids to occur;

detecting expression of such nucleic acids.

In a preferred embodiment, the agent induces NFAT signaling, and/or inhibits or reduces NFAT-NFAT ligand signaling. Preferably, the expression of the anergy-associated nucleic acids is increased.

In a preferred embodiment, the agent inhibits or reduces NFAT signaling, and/activates NFAT-NFAT ligand signaling. Preferably, the expression of the anergy-associated nucleic acids is decreased.

In another aspect, the invention features, a method of inducing or promoting anergy. The method includes:

introducing into a cell a nucleic acid encoding a mutant NFAT polypeptide, e.g., an NFAT mutant unable to interact with, e.g., bind to, an NFAT ligand, e.g., AP-1; and

maintaining the cell under conditions such that the NFAT mutant polypeptide is expressed, thereby reducing or inhibiting the formation of an NFAT-NFAT ligand complex, e.g., an NFAT-AP1 complex, and inducing or promoting anergy.

The subject method can be used on cell-free conditions (e.g., a reconstituted system), on cells in culture, e.g. *in vitro* or *ex vivo* (e.g., cultures comprising immune cells, e.g., T cells). For example, cells can be cultured *in vitro* in culture medium and a peptide, or a nucleic acid, as described herein, can be introduced to the culture medium. In other embodiment, the cells, e.g., the T cells are removed from the subject prior to introducing the nucleic acid. The modified cells can then be returned to the subject. Alternatively, the method can be performed on cells present in a subject, e.g., as part of an *in vivo* (e.g., therapeutic or prophylactic) therapy protocol.

In a preferred embodiment, the mutant NFAT polypeptide is expressed to a greater level than the endogenous NFAT of the cell, e.g., 2, 4, 6, 8, 10 or more fold greater. The mutant NFAT polypeptide is able to bind to nucleic acid, e.g., an NFAT DNA binding site, to a similar extent as the endogenous NFAT. Preferably, the nucleic acid encoding the mutant NFAT polypeptide is operably linked to an inducible promoter, e.g., a promoter that can be regulated by a small molecule, e.g., an organic molecule of molecular weight about 2 000 Daltons or less; or a hormone- or growth factor- specific promoter.

The nucleic acid can be introduced using a gene therapy vector as described below. The gene therapy vector can be delivered to a cell by a membrane bound structure (e.g., a liposome) or a virus (e.g., a retrovirus, herpes virus, or adenovirus).

In a preferred embodiment, the NFAT mutant polypeptide has one or more mutations at positions arginine 468, threonine 535, phenylalanine 475, and/or isoleucine 469 of murine NFAT or at the corresponding positions in human NFAT.

In another aspect, the invention features, a method of evaluating, or identifying, an agent, e.g., an agent as described herein (e.g., a polypeptide, peptide, a peptide fragment, a peptidomimetic, a small molecule), for the ability to modulate, e.g. inhibit, an interaction, e.g., binding, between NFAT-1 and an NFAT ligand (e.g., AP-1). The method includes:

providing a test agent, an NFAT protein or an NFAT ligand-binding fragment thereof, and an NFAT ligand, e.g., an AP-1 complex, or an NFAT-binding fragment thereof;

contacting said test agent, said NFAT protein or fragment thereof, and said NFAT ligand or fragment thereof, under conditions that allow an interaction between NFAT and the NFAT ligand to occur; and

determining whether said test agent modulates, e.g., inhibits, the interaction between said NFAT protein or fragment thereof, and said NFAT ligand or fragment thereof,

wherein a change, e.g., a decrease, in the level of binding between said NFAT protein or fragment thereof, and said NFAT ligand or fragment thereof, is indicative of a modulation, e.g., inhibition, of the interaction between NFAT an NFAT ligand.

In preferred embodiments, the NFAT protein is selected from the group consisting of: NFAT1, NFAT2, NFAT3, and NFAT4.

In preferred embodiments, the test agent is a member of a combinatorial library, e.g., a peptide or organic combinatorial library, or a natural product library.

In a preferred embodiment, a plurality of test compounds, e.g., library members, is tested. In a preferred embodiment, the plurality of test compounds, e.g., library members, includes at least 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 compounds. In a preferred embodiment, the plurality of test compounds, e.g., library members, share a structural or functional characteristic.

In a preferred embodiment, test compound is a peptide or a small organic molecule.

In a preferred embodiment, the method is performed in cell-free conditions (e.g., a reconstituted system).

In a preferred embodiment, the method further includes: contacting said agent with a test cell, or a test animal, to evaluate the effect of the test agent on the interaction between NFAT and an NFAT ligand (e.g., AP-1).

In a preferred embodiment, the ability of the agent to modulate an interaction, e.g., binding, between NFAT-1 and an NFAT ligand is evaluated in a second system, e.g., a cell-free, cell-based, or an animal system.

In a preferred embodiment, the ability of the agent to modulate an interaction, e.g., binding, between NFAT-1 and an NFAT ligand is evaluated in a cell based system, e.g., a two-hybrid assay.

In another aspect, the invention features, a method of evaluating, or identifying, an agent, e.g., an agent as described herein (e.g., a polypeptide, peptide, a peptide fragment, a peptidomimetic, a small molecule), for the ability to modulate, e.g. enhance or decrease, transcription of an anergy-associated nucleic acid. The method includes:

contacting a cell (e.g., an immune cell, e.g., a T- or a B- cell or cell line), or an NFAT-containing transcription complex, with a test agent; and

determining whether said test agent modulates, e.g., activates, transcription of at least one anergy-associated nucleic acid, wherein a change, e.g., an increase, in the level of expression of said anergy-associated nucleic acid is indicative of a modulation, e.g., activation, of the expression of anergy-associated nucleic acids.

In a preferred embodiment, the level of expression of at least one, two, three or four anergy-associated nucleic acid is evaluated

In a preferred embodiment, the level of expression of the at least one anergy-associated nucleic acid is evaluated after stimulation of the cell, e.g., the immune cell, with an antigen.

In preferred embodiments, the test agent is a member of a combinatorial library, e.g., a peptide or organic combinatorial library, or a natural product library.

In a preferred embodiment, a plurality of test compounds, e.g., library members, is tested. In a preferred embodiment, the plurality of test compounds, e.g., library members, includes at least 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 compounds.

In a preferred embodiment, the plurality of test compounds, e.g., library members, share a structural or functional characteristic.

In a preferred embodiment, test compound is a peptide or a small organic molecule.

In a preferred embodiment, the method is performed in cell-free conditions (e.g., a reconstituted system).

In a preferred embodiment, the method is performed in a cell (e.g., a T- or a B-cell or cell line).

In preferred embodiments, the NFAT is selected from the group consisting of: NFAT1, NFAT2, NFAT3, and NFAT4.

In a preferred embodiment, the method further includes: contacting said agent with a test cell, or a test animal, to evaluate the effect of the test agent on the transcription of the anergy-associated nucleic acid.

In a preferred embodiment, the ability of the agent to modulate transcription of the anergy-associated nucleic acid is evaluated in a second system, e.g., a cell-free, cell-based, or an animal system.

In a preferred embodiment, the ability of the agent to modulate transcription of the anergy-associated nucleic acid is evaluated in a cell-based system, e.g., a two-hybrid assay.

Also within the scope of the invention are agents identified using the methods described herein.

In another aspect, the invention features, a pharmaceutical composition comprising an amount of an agent as described herein, and a pharmaceutically acceptable carrier. In one embodiment, the compositions of the invention, e.g., the pharmaceutical compositions, are administered in combination therapy, i.e., combined with other agents, e.g., therapeutic agents, that are useful for treating disorders, such as cancers, immune cell- mediated disorders, or infections.

In another aspect, the invention features, a method of analyzing NFAT-mediated gene expression. This method can be used to identify nucleic acids having

an expression which is modulated by imbalanced activation of NFAT, relative to the NFAT-NFAT ligand or NFAT ligand alone. Such nucleic acids are candidates for inducing, maintaining, and/or propagating an anergic or tolerant state (also referred to herein as "anergy-associated nucleic acids" or " anergy-associated genes"). The method includes:

providing nucleic acids (e.g., RNAs or cDNAs), or gene products (e.g., proteins), expressed in a cell (e.g., an immune cell) in which anergy has been induced; and

analyzing nucleic acids, or gene products, expressed in said cell, compared to the expression of the same nucleic acids, or gene products, in a cell in which anergy has not been induced (e.g., untreated cells), or in which immune activity has been modulated differently (e.g., cells treated with an immunosuppressant, e.g., cyclosporine A or FK506), thereby identifying nucleic acids or gene products which are modulated by NFAT imbalanced activation.

In a preferred embodiment, the cell is an immune cell, e.g., a T or a B cell or cell line.

In a preferred embodiment, the interaction, e.g., binding, between NFAT and NFAT-ligand (e.g., AP-1) is inhibited by, for example, selectively activating expression and/or activity of NFAT relative to an NFAT-ligand (e.g., AP-1). For example, NFAT activity or expression can be selectively induced by contacting a cell, e.g., an immune cell, e.g., a T cell, with an agonist that selectively activates NFAT activity or expression, relative to AP-1. Exemplary agonists include a T cell receptor ligand (e.g., an antigen or an antibody), and a calcium ionophore (e.g., ionomycin).

In a preferred embodiment, anergy is induced by inhibiting an interaction, e.g., binding, between NFAT and an NFAT ligand (e.g., AP-1). Preferably, the interaction, e.g., binding, between NFAT and NFAT-ligand (e.g., AP-1) is inhibited by introducing into a cell (e.g., an immune cell) a mutant NFAT protein, which has been modified to disrupt at least one amino acid residue that makes critical contact with an AP-1 component (e.g., Fos and/or Jun). Preferably, the mutant NFAT protein includes at least one amino acid substitution, deletion and/or insertion. Preferably, the mutant NFAT includes at least one, preferably two, and most preferably three amino acid substitutions. Most preferably, the amino acid substitutions involve amino acid

residues that make critical contact with Fos and Jun (e.g., at least one of: arginine at position 466, isoleucine at position 467, and threonine at position 533). Most preferably, the mutant NFAT includes at least one arginine to alanine substitution at position 466, isoleucine to alanine substitution at position 467, and threonine to glycine substitution at position 533. The gene expression in a cell transfected with mutant NFAT can be compared to a control, e.g., a cell transfected with wild type NFAT.

In a preferred embodiment, anergy is induced by inhibiting the interaction, e.g., binding, between NFAT and an upstream NFAT activator (e.g., calcineurin), for example, by introducing into a cell (e.g., an immune cell) a selective NFAT-calcineurin inhibitor, e.g., a VIVIT peptide or an analog thereof (e.g., a peptidomimetic). Preferably, the selective NFAT inhibitor is expressed under inducible control.

In a preferred embodiment, the nucleic acids or gene products from an NFAT-expressing cell (e.g., an NFAT-expressing T cell) are compared to the nucleic acids or gene products from an NFAT-negative cell (e.g., an NFAT-negative T cell). The method further includes: contacting said NFAT-expressing cell and NFAT-negative cell with an NFAT inhibitor, e.g., a VIVIT peptide or an analog thereof, and determining the changes in gene expression, wherein a modulation (e.g., a decrease) in the expression of the nucleic acids or gene products in the presence of the VIVT peptide is indicative of a nucleic acid under NFAT control, e.g., a candidate anergy-associated nucleic acid or gene product.

In a preferred embodiment, the cells (e.g., the T cells) are stimulated with a calcium ionophore, e.g., ionomycin; control cells are contacted with a control solution. Calcium ionophore -stimulated and control cells may or may not express VIVIT. Fully-stimulated cells (e.g., T cells) (PMA+ionomycin, anti-CD3+anti-CD28 or antigen/APC) expressing or not expressing VIVIT can also be used as a control. The difference between these results will identify NFAT-dependent nucleic acids that are turned on in the presence versus absence of an NFAT-ligand (e.g., AP-1, NFκB).

In a preferred embodiment, nucleic acids (e.g., RNAs or cDNAs) from the cell are analyzed by one or more of: DNA arrays, subtractive strategies, or genome-wide retroviral insertion.

In a preferred embodiment, nucleic acids (e.g., RNAs or cDNAs) from a cell is contacted with an array having a plurality of probes, wherein each of said probes of the plurality is positionally distinguished from the other probes in the plurality, thereby identifying nucleic acids which are modulated by modulation of an NFAT-NFAT ligand interaction.

In another aspect, the invention features an array. The array includes a substrate having a plurality of addresses. Each address of the plurality includes a capture probe, e.g., a unique capture probe. Preferably, an address has a single species of capture probe, e.g., each address recognizes a single species. The addresses can be disposed on the substrate in a two-dimensional or three-dimensional configuration.

In a preferred embodiment, at least one address of the plurality includes a capture probe that hybridizes specifically to an anergy-associated nucleic acid selected from the group consisting of e.g., a protein involved in a proteolytic program (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1))

In a preferred embodiment, the plurality of addresses includes two, three, four, five or six addresses having nucleic acid capture probes for the anergy-associated nucleic acids. In a preferred embodiment, the plurality of addresses includes addresses having capture probes for all six anergy-associated nucleic acids. Most preferably, the plurality of addresses includes addresses having capture probes for TNF-α and IL-13.

In another preferred embodiment, at least one address of the plurality includes a capture probe that binds specifically to a polypeptide selected from the group of polypeptides encoded by an anergy-associated nucleic acid. Preferably, the capture probe is an antibody or derivative thereof. In another preferred embodiment, the plurality of addresses includes two, three, four, five, or six addresses having

polypeptide capture probes for different polypeptides encoded by the anergy-associated nucleic acids.

In another aspect, the invention features a first method of evaluating a sample. The method includes determining the expression of at least one anergy-associated nucleic acid, e.g., a value for the expression of at least one anergy-associated nucleic acid; and comparing the expression, e.g., the value, to a reference, e.g., a reference value, to thereby evaluate the sample. A change in the expression, e.g., value, relative to the reference, e.g., the reference value, is an indication that the sample differs from a sample used to obtain the reference, e.g., the reference value. The expression, e.g., value, can be a qualitative or quantitative assessment of the abundance of 1) an mRNA transcribed from the nucleic acid, or of 2) the polypeptide encoded by the nucleic acid.

In a preferred embodiment, the reference value can be obtained by determining a value of the expression of the nucleic acid in a normal sample, a diseased sample, or an anergic immune cell (e.g., T or B cell) population.

In a preferred embodiment, the expression, e.g., a value for expression, can be determined by quantitative PCR, Northern blotting analysis, microarray analysis, serial analysis of gene expression, and other routine methods.

In a preferred embodiment, the anergy-associated nucleic acid is selected from the group consisting of: e.g., a protein involved in a proteolytic program (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1)).

In another aspect, the invention features a second method of evaluating a sample. The method includes providing a sample expression profile and at least one reference expression profile; and comparing the sample expression profile to at least one reference expression profile to thereby evaluate the sample.

In a preferred embodiment, an expression profile includes a plurality of values, wherein each value corresponds to the level of expression of a different gene, splice-variant or allelic variant of a nucleic acid or a translation product thereof. The value can be a qualitative or quantitative assessment of the level of expression of the nucleic acid or the translation product of the nucleic acid, i.e., an assessment of the abundance of 1) an mRNA transcribed from the nucleic acid, or of 2) the polypeptide encoded by the nucleic acid.

In a preferred embodiment, the sample expression profile and the reference profile have a plurality of values, one or more of which correspond to an anergy-associated nucleic acid.

In a preferred embodiment, the profiles include values for two, three, four, five or all six nucleic acids of the group. Preferably, the profiles include additional values for nucleic acids that are not members of the group. Most preferably, the profiles include values for TNF-α and IL-13.

In a preferred embodiment, an increase in the expression level of TNF-α and IL-13, and no substantial change in the expression level of: IL-2, IFN-γ, IL-10, and MIP-1α is an indication that the sample is in an anergic state.

In a preferred embodiment, a plurality of reference profiles is provided. A reference profile can be a profile obtained from a normal sample, a diseased sample, or an anergic immune cell (e.g., T or B cell) population. A reference profile can also be an expression profile obtained from any suitable standard, e.g., a nucleic acid mixture.

In one preferred embodiment, the sample expression profile is compared to a reference profile to produce a difference profile. In a preferred embodiment, the sample expression profile is compared indirectly to the reference profile. For example, the sample expression profile is compared in multi-dimensional space to a cluster of reference profiles.

In a preferred embodiment, the sample expression profile is obtained from an array. For example, the method further includes providing an array as described above; contacting the array with a nucleic acid mixture (e.g., a mixture of nucleic acids obtained or amplified from a cell), and detecting binding of the nucleic acid mixture to the array to produce a sample expression profile. In another embodiment,

the sample expression profile is determined using a method and/or apparatus that does not require an array (e.g., SAGE or quantitative PCR with multiple primers)

The method can further include harvesting mRNA from the sample and reverse transcribing the mRNA to produce cDNA, e.g., labeled or unlabelled cDNA. Optionally, the cDNA can be amplified, e.g., by a thermal cycling (e.g., polymerase chain reaction (PCR)) or an isothermal reaction (e.g., NASBA) to produce amplified nucleic acid for use as the nucleic acid mixture that is contacted to the array.

In one embodiment, the sample is a blood sample, a spleen sample, or a lymph sample. Preferably the sample includes immune cells (T cells or B cells). In a preferred embodiment, the method further includes fluorescent-activated cell sorting (FACS) of the sample prior to harvesting mRNA. For example, FACS can be used to isolate a subtype of T cells, e.g., Th1 cells, T cells with a particular T-cell receptor, T cells of various stages of maturation, helper T cells, killer T cells, and the like.

Also featured is a method of evaluating a subject. The method includes providing a sample from the subject and determining a sample expression profile, wherein the profile includes a value representing the level of expression of anergy-associated nucleic acids (e.g. selected from the group consisting of: a protein involved in a proteolytic program (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1)).

In a preferred embodiment, the profiles include values for two, three, four, five or all six nucleic acids of the group. Preferably, the profiles include additional values for nucleic acids that are not members of the group. Most preferably, the profiles include values for TNF-α and IL-13.

In a preferred embodiment, an increase in the expression level of TNF-α and IL-13, and no substantial change in the expression level of: IL-2, IFN-γ, IL-10, and MIP-1α is an indication that the sample is in an anergic state.

The method can further include comparing the value or the profile (i.e., multiple values) to a reference value or reference profile. The expression profile of the sample can be obtained as described above. The method can be used to a) diagnose an immune (e.g., a T cell) disorder in a subject; b) monitor an infection, e.g., a viral, bacterial, fungal, or parasitic infection in a subject; and c) monitor immunosuppression therapy in a subject (e.g., prior to, during, or following transplantation, or administration of cyclosporin A and FK506). For example, the immune cell disorder can be myocardial hypertrophy, allergy, arthritis, and autoimmune disease.

An alteration in the expression of an anergy-associated nucleic acid is an indication that the subject has or is disposed to having an immune (e.g., a T or B) cell disorder, e.g., anergy or an immunocompromised disorder. Preferably, expression of a plurality (e.g., two, three, four, five, or six of anergy-associated nucleic acids is altered. In one embodiment, an increase in the expression level of TNF-α and IL-13, and no substantial change in the expression level of IL-2, IFN-γ, IL-10, and MIP-1α is an indication of the subject having immune (e.g., T or B) cells in an anergic state.

The method can be used to monitor a treatment for an immune cell disorder (e.g., T cell anergy or T cell hyperstimulation) in a subject. For example, a subject expression profile can be determined in a subject during treatment. The subject expression profile can be compared to a reference profile or to a profile obtained from the subject prior to treatment or prior to onset of the disorder. In a preferred embodiment, the subject expression profile is determined at intervals (e.g., regular intervals) during treatment.

The treatment can be a treatment that inhibits calcineurin activity e.g., treatment with cyclosporin A or FK506. The treatment can be with a specific NFAT inhibitor (see, e.g., Aramburu *et al.* (1999) *Science* 285:2129).

In another aspect, the invention features a transactional method of evaluating a subject. The method includes: a) obtaining a sample from a caregiver; b) determining a subject expression profile for the sample; and c) transmitting a result to the caregiver.

Optionally, the method further includes either or both of steps: c) comparing the subject expression profile to one or more reference expression profiles; and d) selecting the reference profile most similar to the subject reference profile. The reference expression profiles can be include one or more of: i) a profile from a like sample from a normal subject; ii) a profile from a like sample from a subject having a disease or disorder (e.g., a T cell disorder, an autoimmune disease, an immune-compromised state); iii) a profile from a like sample from a subject having a disease or disorder and undergoing a treatment; and iv) a profile from the subject being evaluated, e.g., an earlier profile or a normal profile of the same subject.

The result transmitted to the caregiver can be one or more of: information about the subject expression profile, e.g., raw or processed expression profile data and/or a graphical representation of the profile; a difference expression profile obtained by comparing the subject expression profile to a reference profile; a descriptor of the most similar reference profile; the most similar reference profile; and a diagnosis or treatment associated with the most similar reference profile. The result can be transmitted across a computer network, e.g., the result can be in the form of a computer transmission (e.g., across the Internet or a private network, e.g., a virtual private network). The result can be transmitted across a telecommunications network, e.g., using a telephone or mobile phone. The results can compressed and/or encrypted.

The expression profiles can be determined, e.g., using an array (e.g., a nucleic acid or polypeptide array) as described herein or using a method and/or apparatus that does not require an array (e.g., SAGE or quantitative PCR with multiple primers)

In a preferred embodiment, the subject expression profile and the reference profiles include a value representing the level of expression of one or more anergy-associated nucleic acids. In a preferred embodiment, the profiles include values for two, three, four, five or all six nucleic acids of the group. Preferably, the profiles

include additional values for nucleic acids that are not members of the group. Most preferably, the profiles include values for TNF-α and IL-13.

In a preferred embodiment, an increase in the expression level of TNF- α and IL-13, and no substantial change in the expression level of IL-2, IFN-γ, IL-10, and MIP-1α is an indication of the subject having immune cells in an anergic state.

In the context of expression profiles herein, "most similar" refers to a profile, which for more than one value of the profile, compares favorably to a given profile. A variety of routine statistical measures can be used to compare two reference profiles. One possible metric is the length (i.e. Euclidean distance) of a difference vector representing the difference between the two profiles. Each of the subject and reference profile is represented as a multi-dimensional vector, wherein the coordinate of each dimension is a value in the profile. The distance of the difference vector is calculated using standard vectorial mathematics. In another embodiment, values for different nucleic acids in the profile are weighted for comparison.

Also featured is a computer medium having encoded thereon computer-readable instructions to effect the following steps: receive a subject expression profile; access a database of reference expression profiles; and either i) select a matching reference profile most similar to the subject expression profile or ii) determine at least one comparison score for the similarity of the subject expression profile to at least one reference profile. The subject expression profile, and the reference expression profiles each include values representing the level of anergy-associated nucleic acid expression (e.g., one or more anergy-associated nucleic acids described herein).

In a preferred embodiment, the subject expression profile and the reference profiles include a value representing the level of expression of one or more anergy-associated nucleic acids. In a preferred embodiment, the profiles include values for two, three, four, five, or all six nucleic acids of the group. Preferably, the profiles include additional values for nucleic acids that are not members of the group. Most preferably, the profiles include values for TNF-α and IL-13.

In another aspect, the invention features a computer medium having a plurality of digitally encoded data records. Each data record includes values representing the level of expression of anergy-associated gene expression (e.g., one or more anergy-associated nucleic acids described herein) in a sample, and a descriptor of the sample. The descriptor of the sample can be an identifier of the sample, a subject from which the sample was derived (e.g., a patient), a diagnosis (e.g., a T cell disorder, an immunodeficiency, an autoimmune disease or an infection), or a treatment (e.g., a preferred treatment, an immunosuppressant). In a preferred embodiment, the records include records for one or more samples from a normal individual, an abnormal individual (e.g., an individual having a disease or disorder), and *in vitro* culture T cells. The abnormal individual can be an immune-compromised individual (e.g., an AIDS patient, an individual treated with an immunosuppressant (e.g., FK506, cyclosporin A)), an individual having an infection (e.g., viral, bacterial, fungal, or parasitic infection), an individual exposed to a superantigen, or an individual having an autoimmune disease. *In vitro* cultured T cells can include T cells exposed *in vitro* to a drug (e.g., cyclosporin A or FK506), an antigen presenting cell, a cytokine, or a virus.

In a preferred embodiment, the data record further includes values representing the level of expression of nucleic acids other than anergy-associated nucleic acids. In one embodiment, the data record further includes a value representing the level of expression for each nucleic acid detected by a capture probe on an array described herein.

In a preferred embodiment, the subject expression profile and the reference profiles include a value representing the level of expression of one or more anergy-associated nucleic acids, e.g., one or more anergy-associated nucleic acids as described herein.

In a preferred embodiment, the profiles include values for two, three, four, five, or all six nucleic acids of the group. Preferably, the profiles include additional values for nucleic acids that are not members of the group. Most preferably, the profiles include values for TNF-α and IL-13.

In another aspect, the invention features a method of evaluating, or identifying, an agent, e.g., a compound that alters an immune cell activity (e.g., a compound that induces anergy or a compound that stimulates immune cells to exit anergy). The method includes: providing one or more reference profiles; contacting the agent to an immune (e.g., a T or B) cell; determining a agent-associated expression profile, e.g., using a method described herein; and comparing the agent-associated expression profile to at least one reference profile.

The agent-associated expression profile and the reference profiles include a value representing the level of expression of one or more anergy-associated nucleic acids, e.g., one or more anergy-associated nucleic acids as described herein.

In a preferred embodiment, the profiles include values for two, three, four, five, or all six nucleic acids of the group. Preferably, the profiles include additional values for nucleic acids that are not members of the group. Most preferably, the profiles include values for TNF-α and IL-13.

In a preferred embodiment, an increase in the expression level of TNF-α and IL-13, and no substantial change in the expression level of IL-2, IFN-γ, IL-10, and MIP-1 α is an indication that the compound induces anergy.

In one embodiment, the reference profiles include one or more of a profile of an immune cell (e.g., a T cell) in an anergic state, a profile of an immune cell (e.g., a T cell) in a normal state, and a profile of an immune cell (e.g., a T cell) in an activated state. In a preferred embodiment, the contacted immune cell is in an anergic state. For example, prior, during, or after the immune cell is contacted with the test agent, the immune cell can be contacted with cyclosporin A or FK506.

In another preferred embodiment, the method further includes, e.g., prior to determining the expression profile, contacting an immune cell with an antigen and/or an antigen presenting cell, e.g., to stimulate the immune cell with antigen. The compound-associated expression profile can be determined at periodic intervals after contact with the antigen.

In another preferred embodiment, the contacted immune cell is in a normal state.

In still another preferred embodiment, the contacted immune cell is in an activated state (e.g., activated by a phorbol ester, a cytokine, or an antigen presenting cell).

In a preferred embodiment, the method includes comparing the agent expression profile to a plurality of reference profiles (e.g., all reference profiles), and identifying a most similar reference profile as an indication of the efficacy and/or utility of the agent. In another preferred embodiment, multiple compound-associated expression profiles are determined at periodic intervals after contact with the agent.

In another aspect, the invention features a method of evaluating, or identifying, an agent, e.g., a compound that alters an immune cell (e.g., a T cell) state, e.g., enhances entry into or exit from an anergic state. The method includes: providing a first cell having a first nucleic acid encoding a first reporter gene operably linked to a first promoter that includes an NFAT binding site (e.g., an AP-1 independent κB-like site, e.g., the κ3 site of the TNFα promoter); contacting the cell with the agent; and detecting the presence of the polypeptide encoded by the first reporter gene. A change in the level of expression of the first reporter gene is an indication that the agent can alter a T cell state.

In one embodiment, the method further includes providing a second cell having a nucleic acid encoding a second reporter gene (the same or different from the first reporter gene) operably linked to a second promoter that includes a composite NFAT-AP1 binding site (e.g., ARRE-2); and detecting the presence of the polypeptide encoded by the second reporter gene. The expression level of the first and the second reporter gene can be compared. An agent that decreases the level of the second reporter gene relative to the first reporter gene is indicated as a compound that enhances entry into an anergic state. A compound that increases the level of the second reporter gene relative to the first reporter gene is indicated as a compound that enhances exit from an anergic state.

In another embodiment, the method further includes treating the cell with a calcium ionophore, e.g., ionomycin, and an NFAT ligand activator, e.g., PMA, prior to contacting the cell with the agent. An agent that decreases the level of the second reporter gene, but not the first reporter gene is indicated as a compound that enhances

entry into an anergic state. An agent that increases the level of the second reporter gene relative to the first reporter gene is indicated as a compound that enhances exit from an anergic state.

In another embodiment, the first cell also has a second nucleic acid encoding a second reporter gene operably linked to a second promoter that includes a composite NFAT-NFAT ligand (e.g., NFAT-AP1) binding site (e.g., ARRE-2). The second reporter gene differs from the first reporter gene. The expression level of the second reporter gene can be detected prior, concurrent, or after detection of the expression level of the first reporter gene.

In another aspect, the invention features, a method of evaluating, or identifying an agent, e.g., a compound, that alters immune cell (e.g.. T or B cell) state, e.g., enhances entry into or exit from an anergic state. The method includes: providing a first cell having a nucleic acid encoding a first reporter gene operably linked to a regulatory region of an anergy-associated nucleic acid, e.g., an anergy-associated nucleic acid as described herein; contacting the cell with the agent; and detecting the presence of the polypeptide encoded by the first reporter gene.

In one embodiment, the method further includes providing a second cell having a nucleic acid encoding a second reporter gene (the same or different from the first reporter gene) operably linked to a second promoter that includes a composite NFAT-NFAT ligand (e.g., NFAT-AP1) binding site (e.g., ARRE-2); and detecting the presence of the polypeptide encoded by the second reporter gene. The expression level of the first and the second reporter gene can be compared. An agent that decreases the level of the second reporter gene relative to the first reporter gene is indicated as a compound that enhances entry into an anergic state. An agent that increases the level of the second reporter gene relative to the first reporter gene is indicated as a compound that enhances exit from an anergic state.

In another aspect, the invention features, a purified nucleic acid, and the purified protein product, of a nucleic acid discovered by a method described herein. Such nucleic acids or proteins can be used to discover further downstream expressed nucleic acids or to screen for molecules which modulate an immune response.

In another aspect, the invention features, a nucleic acid which encodes a mutant NFAT as described herein, vectors encoding said mutant nucleic acid, cells transformed with said vector, and the corresponding mutant protein. These reagents are useful in drug screening methods as described herein.

In preferred embodiments, the mutant NFAT protein includes at least one amino acid substitution, deletion and/or insertion. Preferably, the mutant NFAT includes at least one, preferably two, and most preferably three amino acid substitutions. Preferably, the amino acid substitutions involve amino acid residues that make critical contact with Fos and Jun (e.g., at least one of: arginine at position 466, isoleucine at position 467, and threonine at position 533). Most preferably, the mutant NFAT includes at least one of arginine to alanine substitution at position 466, isoleucine to alanine substitution at position 467, and threonine to glycine substitution at position 533.

Other features and advantages of the instant invention will become more apparent from the following detailed description and claims.

Brief Description of the Drawings

Figures 1A-1B are schematic representations of the mechanism of normal T cell signaling (Figure 1A) and tolerance induction (Figure 1B). Note that some overlap is possible, i.e. some "anergy-associated nucleic acids" may also be induced during the productive immune response, and some nucleic acids associated with the productive immune response, e.g. cytokine nucleic acids, may be be induced during the process of anergy induction.

Figure 2 is a graph depicting the decrease in proliferative response of antigen presenting cells (APC) to antigen after pretreatment of D5 T cells with ionomycin.

Figure 3 is a bar graph showing impaired anergy induction in Th1 cells from NFAT1-/- mice.

Figure 4 is a bar graph showing that NFAT1-/- Th1 cells were effectively insusceptible to anergy induced by anti-CD3.

Figure 5 is a bar graph showing that inclusion of CsA during pretreatment with immobilized anti-CD3 prevents the induction of anergy.

Figure 6 is a FACS analysis of retroviral transduction of NFAT1 into primary CD4+ T cells from NFAT1-/- mice.

Figure 7 is a bar graph showing similar transactivation of κ3-CAT and TNFα-promoter-Luc plasmids but impaired transactivation of NFAT-AP-1 and IL2-promoter.

Figure 8 is a bar graph showing that overexpression of wildtype NFAT1, but not the AP-1-less mutant of NFAT1, enhances activation-induced cell death.

Detailed Description of the Invention

The present invention is based, in part, on the theory that tolerance results from imbalanced activation of the calcium calcineurin-dependent transcription factor NFAT, relative to the CD28-activated transcription factors AP-1 (Fos/Jun, Jun/Jun) and NFκB/Rel. NFAT is activated by calcium mobilization via the T cell receptor (TCR) (reviewed in [Rao, A et al. (1997) *Annu Rev of Immunol.* 5: 707-749; Crabtree, G.R. (1999) *Cell* 96: 611-614), while cJun, RelA and cRel are maximally activated by ligation of both TCR and CD28 (Su, B. et al. (1994) *Cell* 77: 727-736; Harhaj, E.W. et al. *J Biol Chem* 273: 25185-25190; Himes S.R et al. (1996) *Immunity* 5: 479-489; Ghosh P et al. (1992) *Proc Natl Acad Sci* USA 90: 1696-1700). Under conditions of full stimulation through both the TCR and CD28, activation of NFAT, AP-1, NFκB/Rel and other transcription factors results in transcription of the cytokine nucleic acids and other nucleic acids associated with a productive immune response (Rao, A et al. (1997) *supra*). A schematic representation of the mechanism of normal T cell activation and tolerance induction is shown is Figures 1A-1B, respectively). In contrast, when T cells are stimulated with ionomycin alone, or when they are stimulated through the TCR, in the absence of CD28 stimulation, NFAT becomes activated without significant activation of AP-1 or NFκB (Figure 1B). Applicants believed that imbalanced NFAT activation turns on a distinct genetic program associated with the anergic or tolerant state. Among the nucleic acids turned on under these conditions (also referred to herein as "anergy-associated nucleic acids") there

are some whose products have a negative feedback effect on the production of an immune response, e.g., these nucleic acid products may uncouple an antigen receptor from the proximal signaling pathways.

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

By "NFAT protein" or "NFAT" (nuclear factor of activated T cells) is meant a member of a family of transcription factors comprising the members NFAT1, NFAT2, NFAT3 and NFAT4, with several isoforms. Any other NFAT protein whose activation is calcineurin dependent is also meant to be included. NFAT proteins can be, e.g., mammalian proteins, e.g., human or murine. NFAT1, NFAT2 and NFAT4 are expressed in immune cells, e.g., T lymphocytes, and play a role in eliciting immune responses. NFAT proteins are involved in the transcriptional regulation of cytokine nucleic acids, e.g., IL-2, IL-3, IL-4, TNF-a and IFN-y, during the immune response. cDNA sequences for NFAT have been previously reported. See McCaffrey et al., Science 262: 750-754 (1993) and Luo et al., Mol. Cell Biol. 16: 3955-3966 (1996) for murine NFAT1. & Luo et al., Mol. Cell Biol. 16: 3955-3966 (1996) for human NFAT1. See Northrop et al., Nature 369: 497-502 (1994) for human NFAT2, and Park et al., J. Biol. Chem. 271: 20914-20921 (1996) for human NFAT2b. The published sequences for human NFAT2 represent two isoforms differing by alternative splicing at the N and C termini, but having the same regulatory domain and DNA-binding domain. See Hoey et al., Immunity 2: 461-472 (1995) for human NFAT3. See Masuda et al., Mol. Cell Biol. 15: 2697-2706 (1995) and Hoey et al., Immunity 2: 461-472 (1995) for human NFAT4. See Ho et al., (1995) J. Biol. Chem. 270: 19898-19907 and Liu et al. (1997), Mol. Cell Biol. 8: 157-170 for murine NFAT4. The two published sequences for murine NFAT4 are not identical.

NFAT proteins have been shown to be direct substrates of calcineurin. Calcineurin is a calmodulin-dependent, cyclosporin A/FK506-sensitive, phosphatase. Calcineurin is activated through its interaction with Ca^{+2}activated calmodulin when intracellular calcium levels are elevated as a result of receptor crosslinking and phospholipase C activation. The activated calcineurin in turn activates NFAT from an inactive cytoplasmic pool. NFAT activation involves protein-protein interaction

between calcineurin and NFAT, dephosphorylation of NFAT by calcineurin, conformational change in NFAT resulting from the interaction between calcineurin and NFAT or the dephosphorylation of NFAT and translocation of NFAT to the nucleus. NFAT activation results in induction of gene expression.

The present invention discloses at least two distinct NFAT-mediated genetic programs, referred to herein as "NFAT signaling" and "NFAT-NFAT ligand signaling." "NFAT signaling" or "NFAT-mediated immune response" refers to a calcium-triggered cascade of signal transduction events that leads to NFAT activation, without substantial expression and/or activity of an NFAT ligand, where an "NFAT-ligand" is defined as a protein or transcription factor that interacts physically or functionally with NFATduring the course of a complete or productive immune response. For instance, an NFAT ligand includes the complete set of transcription factors that are turned on during a productive immune response and cooperate physically or functionally with NFAT. Because these transcription factors may also interact physically (e.g. AP-1) or functionally (e.g. NFκB/Rel) with NFAT, they may sometimes be referred to hereafter as "NFAT ligands". Such NFAT-specific activation may result from, e.g., activation of a T cell receptor in the absence of costimulatory receptor stimulation (e.g., CD28 or CD19), or by an increase in intracellular calcium concentration (e.g., using a calcium ionophore, such as ionomycin). These events lead to calcineurin-mediated activation of NFAT. NFAT-specific activation gives rise to expression of one or more "anergy-associated nucleic acids," which may ultimately result in the promotion or induction of anergy or tolerance. A schematic illustration of the NFAT signaling pathway is depicted in Figure 1B. Note that some overlap is possible, i.e. some "anergy-associated nucleic acids" may also be induced during the productive immune response, and some nucleic acids associated with the productive immune response, e.g. cytokine nucleic acids, may be be induced during the process of anergy induction.

"NFAT-NFAT ligand signaling" refers to a cascade of signal transduction events that leads to NFAT and NFAT ligand activation. This coactivation results from, e.g., costimulation of a T or a B cell receptor and a costimulatory receptor (e.g., CD28 or CD19), which in turn activate calcium calcineurin- and protein kinase C-dependent pathways (Figure 1A). The term "NFAT ligand" refers to a protein, or a

complex of proteins (e.g., a protein dimer) that interacts, e.g., binds to, NFAT and leads to a complete productive immune response, including expression of cytokine nucleic acids, cell proliferation, and prevention or minimization of anergy or tolerance. In one embodiment, the NFAT ligand is a CD28-activated transcription factor, such as AP-1 (e.g., Fos/Jun, Jun/Jun dimers, which interact physically with NFAT on specific composite NFAT: AP-1 DNA elements) or NFκB/Rel, which interact functionally with NFAT at other gene regulatory regions.

The costimulatory receptors CD28 and CD19 are present on T and B cells, respectively. CD28 forms a transmembrane homodimer that is present on most T cells and binds to a B7 ligand, e.g., B7-1 (CD80) or B7-2 (CD86), present on antigen presenting cells (APC), such as B cells. B7 family members are typically produced in response to foreign infection. Stimuli that lead to upregulation of B7 proteins include structural components of bacteria, such as lipopolysaccharides, antigen binding to B cells, and tumor necrosis factor α (TNFα). When a T cell is acutely stimulated by an antigen through its T cell receptor, and at the same time costimulated by a B7 protein through the CD28 coreceptor, the combined signal stimulates the T cell to produce IL-2 and to proliferate.

CD19 plays a similar costimulatory role as CD28 in B cells. Like CD28 on T cells, activation of the CD19 costimulatory receptor complex changes the outcome of antigen-receptor ligation. CD19 is a transmembrane protein made constitutively by B cells. On its own, CD19 may act as a receptor for an as yet unidentified ligand, but in association with the complement- binding chain CD21, CD19 forms the signaling subunit of the CR2 complement receptor. The complement system is a proteolytic cascade of interacting serum proteins that is selectively triggered by foreign microorganisms. Once triggered, cleavage products of the third complement component, C3b and C3d, are covalently attached to foreign antigens, tagging them for destruction (C3b) or for immune responses (C3d). When a B cell is acutely stimulated through its B cell receptor and simultaneously costimulated by attached C3d via its complement receptor complex, the combined signal synergistically augments B cell activation and antibody production.

"A costimulatory blocker" or a "costimulatory inhibitor" as used herein, refers to a molecule which binds a member of a ligand/counter-ligand pair (e.g., CD28/B7,

CD19/ligand) and inhibits the interaction between the ligand and counter-ligand or which disrupts the ability of the bound member to transduce a signal. The blocker can be an antibody (or fragment thereof) to the ligand or counter ligand, a soluble ligand (soluble fragment of the counter ligand), a soluble counter ligand (soluble fragment of the counter ligand), or other protein, peptide or other molecule which binds specifically to the counter-ligand or ligand, e.g., a protein or peptide selected by virtue of its ability to bind the ligand or counter ligand in an affinity assay, e.g., a phage display system.

The term "tolerance," as used herein, refers to a down-regulation of at least one element of an immune response, for example, the down-regulation of a humoral, cellular, or both humoral and cellular responses. The term tolerance includes not only complete immunologic tolerance to an antigen, but to partial immunologic tolerance, i.e., a degree of tolerance to an antigen which is greater than what would be seen if a method of the invention were not employed. "Cellular tolerance," or "anergy," refers downregulation of at least one response of an immune cell, e.g., a B a T cell. Such downregulated responses may include: decreased proliferation in response to antigen stimulation; decreased cytokine, e.g., IL-2, production; among others.

As used herein, the term anergy-associated" nucleic acids or their corresponding gene products are those whose expression is modulated (e.g., increased or decreased) in response to NFAT signaling. Changes in the expression of anergy-associated nucleic acids may be a causative factor in inducing, promoting, and/or maintaining tolerance or anergy (i.e., an anergy-inducing nucleic acid), or may simply be a result of the anergic state (i.e., an anergy-induced nucleic acid). Anergy-associated gene products may have a negative feedback on the production of an immune response, e.g., by uncoupling an antigen receptor, e.g., a T or a B cell receptor, from the proximal signaling pathways.

As used herein, the term "nucleic acid molecule" includes DNA molecules (e.g., a cDNA or genomic DNA) and RNA molecules (e.g., an mRNA) and analogs of the DNA or RNA generated, e.g., by the use of nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

Modulators of An NFAT-Mediated Immune Response

The invention provides, in part, drug screen methods to identify agents that modulate an NFAT-mediated immune response (e.g., modulate tolerance or anergy). Such agents can be used, e.g., to modulate an NFAT-NFAT ligand interaction *in vivo* (e.g., as part of therapeutic or prophylactic protocols) or *in vitro* (e.g., in drug screen assays or in assays for identifying anergy associated nucleic acids).

The agent can be an inhibitor of NFAT-NFAT ligand signaling. Such agent can be one or more of: a small molecule (e.g., a chemical agent having a molecular weight of less than 2500 Da, preferably, less than 1500 Da), a polypeptide (e.g., an antibody), a peptide, a peptide fragment, a peptidomimetic, an antisense, a ribozyme, which inhibits NFAT or NFAT ligand expression or activity. Preferably, the agent blocks or reduces an interaction between NFAT and an NFAT-ligand, e.g., AP-1, for example, by binding to the interactive site in NFAT or an NFAT ligand, e.g., AP-1. The agent can be a soluble fragment of NFAT; a soluble fragment of an NFAT ligand, for example, a soluble fragment of an AP-1 component (e.g., a soluble fragment of Fos or Jun); a chemical, e.g., a small organic molecule, e.g., a product of a combinatorial library; a peptide or a polypeptide, e.g., a product of a peptide combinatorial library; a protein or peptide selected in a phage display or other multiple binding-based assay for its ability to inhibit an NFAT-NFAT ligand interaction. In other embodiments, the agent is an antibody that interferes with NFAT-NFAT ligand binding, e.g., a monoclonal antibody, e.g., a human or humanized monoclonal antibody, or an intrabody. In other embodiments, the agent increases NFAT signaling by: increasing NFAT expression, e.g., a nucleic acid encoding NFAT, or an agent which enhances NFAT transcription; and/or decreasing NFAT ligand expression, e.g., an antisense molecule or NFAT ligand ribozyme; an agent which decreases NFAT ligand nucleic acid expression, e.g., a small molecule which binds the promoter of NFAT ligand. In other embodiments, expression of the NFAT-ligand nucleic acids can be decreased by: altering the regulatory sequences of the endogenous NFAT-ligand nucleic acid, e.g., by the addition of a negative regulatory sequence (such as a DNA-biding site for a transcriptional repressor), or by the removal of a positive regulatory sequence (such as an enhancer or a DNA-binding site for a transcriptional activator). Alternatively, NFAT expression can be enhanced

by altering the regulatory sequences of the endogenous NFAT nucleic acid, e.g., by the addition of a positively regulatory sequence (such as an enhancer or a DNA-binding site for a transcriptional activator), or by removal of a negative regulatory sequence (such as a DNA-binding site for a transcriptional repressor).

In those embodiments where tolerance is induced, the agent can be an inhibitor of an upstream activator of an NFAT ligand. In one embodiment, the NFAT ligand inhibitor inhibits or reduces the activity or expression of an NFAT ligand activator, e.g., an activator chosen from protein kinase C (PKC), e.g., protein kinase C theta, MAP kinase, Ras, and Raf. An agent which inhibits an upstream activator of an NFAT ligand can be one or more of: a small molecule which inhibits NFAT ligand expression or activity; an NFAT ligand binding protein which binds to the NFAT ligand, but does not activate the enzyme; an antibody that specifically binds to the NFAT ligand, e.g., an antibody that disrupts NFAT ligand catalytic activity or an antibody that disrupts the ability of upstream activators to activate NFAT ligand; an NFAT ligand nucleic acid molecule which can bind to a cellular NFAT ligand nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein, e.g.. an antisense molecule or NFAT ligand ribozyme; an agent which decreases NFAT ligand nucleic acid expression, e.g., a small molecule which binds the promoter of NFAT ligand. In another preferred embodiment, NFAT ligand is inhibited by decreasing the level of expression of an endogenous NFAT ligand nucleic acid, e.g., by decreasing transcription of the NFAT ligand nucleic acid. In a preferred embodiment, transcription of the NFAT ligand nucleic acid can be decreased by: altering the regulatory sequences of the endogenous NFAT ligand nucleic acid, e.g., by the addition of a negative regulatory sequence (such as a DNA-biding site for a transcriptional repressor), or by the removal of a positive regulatory sequence (such as an enhancer or a DNA-binding site for a transcriptional activator). In another preferred embodiment, NFAT ligand activity is inhibited by a specific small molecule inhibitor. In another preferred embodiment, NFAT ligand activity is inhibited by a monoclonal antibody, e.g., a human or humanized monoclonal antibody.

In those embodiments where tolerance is induced, the agent can be an activator (e.g., increases the activity or the expression) of at least one anergy-associated nucleic acid or protein. Exemplary anergy-associated nucleic acids or

proteins include protein involved in proteolytic programs (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1)).

In those embodiments where tolerance is inhibited or reduced, the agent can be an activator of an upstream activator of an NFAT ligand. In one embodiment, the NFAT ligand activator enhances the activity or expression of an NFAT ligand activator, e.g., an activator chosen from protein kinase C (PKC), e.g., protein kinase C theta, MAP kinase, Ras, and Raf. .

In those embodiments where tolerance is inhibited or reduced, the agent can be an inhibitor (e.g., increases the activity or the expression) of at least one anergy-associated nucleic acid or protein. Exemplary anergy-associated nucleic acids or proteins include protein involved in proteolytic programs (e.g., a protease or RING finger protein (e.g. a caspase or E3 ubiquitin ligase such as Cbl)); a cytokine (e.g., IL-2, IL-13, IFN-γ, TNF-α, or MIP-1α) , especially a cytokine with known immunosuppressive effects (e.g. IL-10, TGFβ); an inhibitory cell surface receptor (e.g. CTLA-4); a signalling protein which downregulates the productive immune response (e.g. serine/threonine or tyrosine phosphatases (e.g., SHP-2); small G proteins and Ras antagonists (e.g., Rap1)). An agent which inhibits an upstream activator of an anergy-associated nucleic acid or protein can be one or more of: a small molecule which inhibits NFAT ligand expression or activity; an anergy-associated protein binding protein which binds to the anergy-associated protein, but does not activate the enzyme; an antibody that specifically binds to the anergy-associated protein, e.g., an antibody that disrupts an anergy-associated protein catalytic activity or an antibody that disrupts the ability of upstream activators to activate anergy-associated nucleic acid protein; a nucleic acid molecule which can bind to a cellular anergy-associated nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein, e.g., an antisense molecule or anergy-associated nucleic acid ribozyme; an agent which decreases NFAT anergy-associated gene expression,

e.g., a small molecule which binds the promoter of anergy-associated nucleic acid. In another preferred embodiment, anergy-associated nucleic acid is inhibited by decreasing the level of expression of an endogenous anergy-associated nucleic acid, e.g., by decreasing transcription of the anergy-associated nucleic acid. In a preferred embodiment, transcription of the anergy-associated nucleic acid can be decreased by: altering the regulatory sequences of the endogenous anergy-associated nucleic acid, e.g., by the addition of a negative regulatory sequence (such as a DNA-biding site for a transcriptional repressor), or by the removal of a positive regulatory sequence (such as an enhancer or a DNA-binding site for a transcriptional activator). In another preferred embodiment, anergy-associated gene activity is inhibited by a specific small molecule inhibitor. In another preferred embodiment, anergy-associated protein activity is inhibited by a monoclonal antibody, e.g., a human or humanized monoclonal antibody, or an intrabody.

Small Molecule Modulators

The modulator of NFAT signaling can be a small molecule (e.g., a chemical agent having a molecular weight of less than 2500 Da, preferably, less than 1500 Da), a chemical, e.g., a small organic molecule, e.g., a product of a combinatorial library. Based on information from the crystal structure of the NFAT1-Fos-Jun-DNA complex (Chen L. et al. (1998) Nature 392: 42-48), critical contacts with Fos and Jun have been identified (see Example 9). Small molecules, e.g., inhibitors, that interact with these critical residues can be designed.

In one embodiment, the invention provides libraries of NFAT or NFAT ligand inhibitors. The synthesis of combinatorial libraries is well known in the art and has been reviewed (see, e.g., E.M. Gordon *et al.*, *J. Med. Chem.* (1994) 37:1385-1401 ; DeWitt, S. H.; Czarnik, A. W. *Acc. Chem. Res.* (1996) 29:114; Armstrong, R. W.; Combs, A. P.; Tempest, P. A.; Brown, S. D.; Keating, T. A. *Acc. Chem. Res.* (1996) 29:123; Ellman, J. A. *Acc. Chem. Res.* (1996) 29:132; Gordon, E. M.; Gallop, M. A.; Patel, D. V. *Acc. Chem. Res.* (1996) 29:144; Lowe, G. *Chem. Soc. Rev.* (1995) 309, Blondelle et al. *Trends Anal. Chem.* (1995) 14:83; Chen et al. *J. Am. Chem. Soc.* (1994) 116:2661; U.S. PAtents 5,359,115, 5,362,899, and 5,288,514; PCT Publication Nos. WO92/10092, WO93/09668, WO91/07087, WO93/20242, WO94/08051).

Libraries of compounds of the invention can be prepared according to a variety of methods, some of which are known in the art. For example, a "split-pool" strategy can be implemented in the following way: beads of a functionalized polymeric support are placed in a plurality of reaction vessels; a variety of polymeric supports suitable for solid-phase peptide synthesis are known, and some are commercially available (for examples, see, e.g., M. Bodansky "Principles of Peptide Synthesis", 2nd edition, Springer-Verlag, Berlin (1993)). To each aliquot of beads is added a solution of a different activated amino acid, and the reactions are allow to proceed to yield a plurality of immobilized amino acids, one in each reaction vessel. The aliquots of derivatized beads are then washed, "pooled" (i.e., recombined), and the pool of beads is again divided, with each aliquot being placed in a separate reaction vessel. Another activated amino acid is then added to each aliquot of beads. The cycle of synthesis is repeated until a desired peptide length is obtained. The amino acid residues added at each synthesis cycle can be randomly selected; alternatively, amino acids can be selected to provide a "biased" library, e.g., a library in which certain portions of the inhibitor are selected non-randomly, e.g., to provide an inhibitor having known structural similarity or homology to a known peptide capable of interacting with an antibody, e.g., the an anti-idiotypic antibody antigen binding site. It will be appreciated that a wide variety of peptidic, peptidomimetic, or non-peptidic compounds can be readily generated in this way.

The "split-pool" strategy results in a library of peptides, e.g., inhibitors, which can be used to prepare a library of test compounds of the invention. In another illustrative synthesis, a "diversomer library" is created by the method of Hobbs DeWitt *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 90:6909 (1993)). Other synthesis methods, including the "tea-bag" technique of Houghten (see, e.g., Houghten *et al.*, *Nature* 354:84-86 (1991)) can also be used to synthesize libraries of compounds according to the subject invention.

Libraries of compounds can be screened to determine whether any members of the library have a desired activity, and, if so, to identify the active species. Methods of screening combinatorial libraries have been described (see, e.g., Gordon *et al.*, *J Med. Chem.*, *supra*). Soluble compound libraries can be screened by affinity chromatography with an appropriate receptor to isolate ligands for the receptor,

followed by identification of the isolated ligands by conventional techniques (e.g., mass spectrometry, NMR, and the like). Immobilized compounds can be screened by contacting the compounds with a soluble receptor; preferably, the soluble receptor is conjugated to a label (e.g., fluorophores, colorimetric enzymes, radioisotopes, luminescent compounds, and the like) that can be detected to indicate ligand binding. Alternatively, immobilized compounds can be selectively released and allowed to diffuse through a membrane to interact with a receptor. Exemplary assays useful for screening the libraries of the invention are described below.

In one embodiment, compounds of the invention can be screened the ability to interact with NFAT or an NFAT ligand by assaying the activity of each compound to bind directly to the NFAT or an NFAT ligand, or to inhibit an interaction between the NFAT or an NFAT ligand, e.g., by incubating the test agent, e.g., test compound, with an NFAT or an NFAT ligand and a lysate, e.g., an immune cell lysate, e.g., in one well of a multiwell plate, such as a standard 96-well microtiter plate. In this embodiment, the activity of each individual compound can be determined. A well or wells having no test compound can be used as a control. After incubation, the activity of each test compound can be determined by assaying each well. Thus, the activities of a plurality of test compounds can be determined in parallel.

In still another embodiment, large numbers of test compounds can be simultaneously tested for binding activity. For example, test compounds can be synthesized on solid resin beads in a "one bead-one compound" synthesis; the compounds can be immobilized on the resin support through a photolabile linker. A plurality of beads (e.g., as many as 100,000 beads or more) can then be combined with yeast cells and sprayed into a plurality of "nano-droplets", in which each droplet includes a single bead (and, therefore, a single test compound). Exposure of the nano-droplets to UV light then results in cleavage of the compounds from the beads. It will be appreciated that this assay format allows the screening of large libraries of test compounds in a rapid format.

Combinatorial libraries of compounds can be synthesized with "tags" to encode the identity of each member of the library (see, e.g., W.C. Still *et al.*, U.S. Patent No. 5,565,324 and PCT Publication Nos. WO 94/08051 and WO 95/28640). In general, this method features the use of inert, but readily detectable, tags, that are

attached to the solid support or to the compounds. When an active compound is detected (e.g., by one of the techniques described above), the identity of the compound is determined by identification of the unique accompanying tag. This tagging method permits the synthesis of large libraries of compounds which can be identified at very low levels. Such a tagging scheme can be useful, e.g., in the "nano-droplet" screening assay described above, to identify compounds released from the beads.

In preferred embodiments, the libraries of transcriptional modulator compounds of the invention contain at least 30 compounds, more preferably at least 100 compounds, and still more preferably at least 500 compounds. In preferred embodiments, the libraries of transcriptional modulator compounds of the invention contain fewer than 10^9 compounds, more preferably fewer than 10^8 compounds, and still more preferably fewer than 10^7 compounds.

NFAT or NFAT Ligand Protein or Peptide Modulators

An NFAT or an NFAT ligand protein or peptide modulators, e.g., inhibitors, can be identified using, e.g., the combinatorial systems described above. Alternatively, fragments or peptides of any of the NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) can be used, e.g., to block interactions among these proteins. The terms "peptide", "polypeptide", and "protein" are used interchangeably herein.

Also within the scope of the invention are variants of the fragments or peptides of any of NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) that include "non-essential" amino acid substitutions. Non-essential amino acid substitutions refer to alterations from the wild-type sequence that can be made without abolishing or more preferably, without substantially altering a biological activity, whereas an "essential" amino acid residue results in such a change.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These

families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) can be preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a coding sequence for NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product), such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity.

In another aspect, the invention also features a modified NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product), e.g., which functions as an agonist (mimetics) or as an antagonist. Preferably the NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) functions as an antagonist of NFAT signaling. For example, an NFAT mutant is described herein which has impaired NFAT ligand binding properties, but retains DNA binding affinity. Variants of the NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) can be generated by mutagenesis, e.g., discrete point mutation, the insertion or deletion of sequences or truncation. An agonist of the NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of the protein. An antagonist of NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) can inhibit one or more of the

activities of the naturally occurring form of the protein by, for example. being capable of binding to a DNA sequence, but incapable of interacting with an NFAT ligand. Thus, specific biological effects can be elicited by treatment with a variant of limited function.

Variants of an NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) can be identified by screening combinatorial libraries of mutants, e.g., truncation mutants, for agonist or antagonist activity.

Libraries of fragments e.g., N terminal, C terminal, or internal fragments, of an NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) sequence can be used to generate a variegated population of fragments for screening and subsequent selection of variants of this protein. Variants in which a phosphorylation site is added or deleted or in which a residue which is glycosylated is added or deleted are particularly preferred.

Methods for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property are known in the art. Recursive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants (Arkin and Yourvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delgrave et al. (1993) *Protein Engineering* 6(3):327-331).

Cell based assays can be exploited to analyze a variegated library. For example, a library of expression vectors can be transfected into a cell line, e.g., a cell line, which ordinarily responds to the protein in a substrate-dependent manner. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of signaling by the NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product)-substrate, and the individual clones further characterized.

In another aspect, the invention features a method of making a mutated NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product), e.g., an NFAT, an NFAT

ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated nucleic acid product) having a non-wild type activity, e.g., an antagonist, agonist, or super agonist of the naturally occurring protein. The method includes: altering the sequence of NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product), e.g., by substitution or deletion of one or more residues of a non-conserved region, a domain or residue disclosed herein, and testing the altered polypeptide for the desired activity.

In another aspect, the invention features a method of making a fragment or analog of a NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product) having an altered biological activity of a naturally occurring protein. The method includes: altering the sequence, e.g., by substitution or deletion of one or more residues, of an NFAT, an NFAT ligand or a component of signaling, e.g., upstream or downstream signaling (e.g., calcineurin, or an anergy-associated gene product), e.g., altering the sequence of a non-conserved region, or a domain or residue described herein, and testing the altered polypeptide for the desired activity.

Antibody Modulators

In other embodiments, the modulators can be an antibody or a fragment thereof, e.g., an antigen binding portion thereof. As used herein, the term "antibody" refers to a protein comprising at least one, and preferably two, heavy (H) chain variable regions (abbreviated herein as VH), and at least one and preferably two light (L) chain variable regions (abbreviated herein as VL). The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" (FR). The extent of the framework region and CDR's has been precisely defined (see, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al. (1987) J. Mol. Biol. 196:901-917, which are incorporated herein by reference). Each VH and VL is

composed of three CDR's and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The antibody can further include a heavy and light chain constant region, to thereby form a heavy and light immunoglobulin chain, respectively. In one embodiment, the antibody is a tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains, wherein the heavy and light immunoglobulin chains are inter-connected by, e.g., disulfide bonds. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. The light chain constant region is comprised of one domain, CL. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The constant regions of the antibodies typically mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (Clq) of the classical complement system.

The term "antigen-binding fragment" of an antibody (or simply "antibody portion," or "fragment"), as used herein, refers to one or more fragments of a full-length antibody that retain the ability to specifically bind to an antigen (e.g., NFAT). Examples of binding fragments encompassed within the term "antigen-binding fragment" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate nucleic acids, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding fragment" of an antibody. These antibody fragments are obtained using conventional techniques known to those with

skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

The antibodies described herein can be human, rodent, humanized, or chimeric antibodies.

Methods of producting antibodies are well known in the art. For example, a monoclonal antibody against a target (e.g., NFAT) can be produced by a variety of techniques, including conventional monoclonal antibody methodology e.g., the standard somatic cell hybridization technique of Kohler and Milstein, Nature 256: 495 (1975). Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed e.g., viral or oncogenic transformation of B lymphocytes. The preferred animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a very well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (e.g., murine myeloma cells) and fusion procedures are also known.

For example, antibodies to phosphorylated or dephosphorylated NFAT peptides can be raised, e.g., by immunization of rabbits e.g., Czernik et al, Methods Enzymol 201: 264-283 (1991) for preparation and characterization of serum or monoclonal antibodies using short synthetic peptides (10-12 residues) corresponding to the sequence surrounding a phosphorylation site. The unphosphorylated peptides can be obtained by conventional methods of chemical synthesis, e.g., Merrifield solid phase synthesis. The phosphopeptides can be obtained, e.g., by in vitro phosphorylation of the synthetic peptides with kinase in instances where the synthetic peptide includes flanking residues that form a consensus site for the kinase (Czernik et al, Methods Enzymol 201: 264-283 (1991)), or, e.g., by chemical synthesis of peptides phosphorylated on serine or threonine residues (Perich JW, Methods Enzymol 201: 225-233 (1991)). The antisera or monoclonal antibodies can be tested to determine whether they show the ability to discriminate between phosphorylated

and unphosphorylated peptides, e.g., by dot immunoblotting or by ELISA (Czernik et al, Methods Enzymol 201: 264-283 (1991)). To ensure that a specific antiserum or monoclonal antibody reagent discriminates between phosphopeptide and dephosphopeptide in the context of NFAT protein, and to select a high-affinity reagent with low background signal in the high-throughput screening assay, the candidate antiserum or monoclonal antibody can be further tested under the conditions to be used in the high-throughput screening assay.

Human monoclonal antibodies (mAbs) directed against human proteins can be generated using transgenic mice carrying the complete human immune system rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, e.g., Wood et al. International Application WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. International Application WO 92/03918; Kay et al. International Application 92/03917; Lonberg, N. et al. 1994 *Nature* 368:856-859; Green, L.L. et al. 1994 *Nature Genet.* 7:13-21; Morrison, S.L. et al. 1994 *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman et al. 1993 *Year Immunol* 7:33-40; Tuaillon et al. 1993 *PNAS* 90:3720-3724; Bruggeman et al. 1991 *Eur J Immunol* 21:1323-1326).

Monoclonal antibodies can also be generated by other methods known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal antibodies (for descriptions of combinatorial antibody display see e.g., Sastry et al. 1989 *PNAS* 86:5728; Huse et al. 1989 *Science* 246:1275; and Orlandi et al. 1989 *PNAS* 86:3833). After immunizing an animal with an immunogen as described above, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known for obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of

the heavy and light chain variable regions from a number of murine antibodies (Larrick et al.,1991, *Biotechniques* 11:152-156). A similar strategy can also been used to amplify human heavy and light chain variable regions from human antibodies (Larrick et al., 1991, *Methods: Companion to Methods in Enzymology* 2:106-110).

In an illustrative embodiment, RNA is isolated from B lymphocytes, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy and light chains are amplified, each alone or in combinantion, and ligated into appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease recognition sequences may also be incorporated into the primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Ideally, the display package comprises a system that allows the sampling of very large variegated antibody display libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating a variegated antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication

WO 93/01288; McCafferty et al. International Publication No. WO 92/01047; Garrard et al. International Publication No. WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

In certain embodiments, the V region domains of heavy and light chains can be expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFV gene subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) 348:552-554, complete V_H and V_L domains of an antibody, joined by a flexible $(Gly_4\text{-}Ser)_3$ linker can be used to produce a single chain antibody which can render the display package separable based on antigen affinity. Isolated scFV antibodies immunoreactive with the antigen can subsequently be formulated into a pharmaceutical preparation for use in the subject method.

Once displayed on the surface of a display package (e.g., filamentous phage), the antibody library is screened with the target antigen, or peptide fragment thereof, to identify and isolate packages that express an antibody having specificity for the target antigen. Nucleic acid encoding the selected antibody can be recovered from the display package (e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques.

Specific antibody molecules with high affinities for a surface protein can be made according to methods known to those in the art, e.g, methods involving screening of libraries (Ladner, R.C., *et al.*, U.S. Patent 5,233,409; Ladner, R.C., *et al.*, U.S. Patent 5,403,484). Further, the methods of these libraries can be used in screens to obtain binding determinants that are mimetics of the structural determinants of antibodies.

In particular, the Fv binding surface of a particular antibody molecule interacts with its target ligand according to principles of protein-protein interactions,

hence sequence data for V_H and V_L (the latter of which may be of the κ or λ chain type) is the basis for protein engineering techniques known to those with skill in the art. Details of the protein surface that comprises the binding determinants can be obtained from antibody sequence information, by a modeling procedure using previously determined three-dimensional structures from other antibodies obtained from NMR studies or crytallographic data. See for example Bajorath, J. and S. Sheriff, 1996, *Proteins: Struct., Funct., and Genet. 24 (2)*, 152-157; Webster, D.M. and A. R. Rees, 1995, "Molecular modeling of antibody-combining sites,"in S. Paul, Ed., *Methods in Molecular Biol. 51*, Antibody Engineering Protocols, Humana Press, Totowa, NJ, pp 17-49; and Johnson, G., Wu, T.T. and E.A. Kabat, 1995, "Seqhunt: A program to screen aligned nucleotide and amino acid sequences," in *Methods in Molecular Biol.51, op. cit.*, pp 1-15.

In one embodiment, a variegated peptide library is expressed by a population of display packages to form a peptide display library. Ideally, the display package comprises a system that allows the sampling of very large variegated peptide display libraries, rapid sorting after each affinity separation round, and easy isolation of the peptide-encoding gene from purified display packages. Peptide display libraries can be in, e.g., prokaryotic organisms and viruses, which can be amplified quickly, are relatively easy to manipulate, and which allows the creation of large number of clones. Preferred display packages include, for example, vegetative bacterial cells, bacterial spores, and most preferably, bacterial viruses (especially DNA viruses). However, the present invention also contemplates the use of eukaryotic cells, including yeast and their spores, as potential display packages. Phage display libraries are described above.

Other techniques include affinity chromatography with an appropriate "receptor", e.g., a target antigen, followed by identification of the isolated binding agents or ligands by conventional techniques (e.g., mass spectrometry and NMR). Preferably, the soluble receptor is conjugated to a label (e.g., fluorophores, colorimetric enzymes, radioisotopes, or luminescent compounds) that can be detected to indicate ligand binding. Alternatively, immobilized compounds can be selectively released and allowed to diffuse through a membrane to interact with a receptor.

The term "modified antibody" is also intended to include antibodies, such as monoclonal antibodies, chimeric antibodies, and humanized antibodies which have been modified by, e.g., deleting, adding, or substituting portions of the antibody. For example, an antibody can be modified by deleting the hinge region, thus generating a monovalent antibody. Any modification is within the scope of the invention so long as the antibody has at least one antigen binding region specific.

Chimeric mouse-human monoclonal antibodies (i.e., chimeric antibodies) can be produced by recombinant DNA techniques known in the art. For example, a gene encoding the Fc constant region of a murine (or other species) monoclonal antibody molecule is digested with restriction enzymes to remove the region encoding the murine Fc, and the equivalent portion of a gene encoding a human Fc constant region is substituted. (see Robinson et al., International Patent Publication PCT/US86/02269; Akira, et al., European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., International Application WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567; Cabilly et al., European Patent Application 125,023; Better et al. (1988 *Science* 240:1041-1043); Liu et al. (1987) *PNAS* 84:3439-3443; Liu et al., 1987, *J. Immunol.* 139:3521-3526; Sun et al. (1987) *PNAS* 84:214-218; Nishimura et al., 1987, *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al., 1988, *J. Natl Cancer Inst.* 80:1553-1559).

The chimeric antibody can be further humanized by replacing sequences of the Fv variable region which are not directly involved in antigen binding with equivalent sequences from human Fv variable regions. General reviews of humanized chimeric antibodies are provided by Morrison, S. L., 1985, *Science* 229:1202-1207 and by Oi et al., 1986, *BioTechniques* 4:214. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of immunoglobulin Fv variable regions from at least one of a heavy or light chain. The recombinant DNA encoding the chimeric antibody, or fragment thereof, can then be cloned into an appropriate expression vector. Suitable humanized antibodies can alternatively be produced by CDR substitution U.S. Patent 5,225,539; Jones et al. 1986 *Nature* 321:552-525; Verhoeyan et al. 1988 *Science* 239:1534; and Beidler et al. 1988 *J. Immunol.* 141:4053-4060.

All of the CDRs of a particular human antibody may be replaced with at least a portion of a non-human CDR or only some of the CDRs may be replaced with non-human CDRs. It is only necessary to replace the number of CDRs required for binding of the humanized antibody to the Fc receptor.

An antibody can be humanized by any method, which is capable of replacing at least a portion of a CDR of a human antibody with a CDR derived from a non-human antibody. Winter describes a method which may be used to prepare the humanized antibodies of the present invention (UK Patent Application GB 2188638A, filed on March 26, 1987), the contents of which is expressly incorporated by reference. The human CDRs may be replaced with non-human CDRs using oligonucleotide site-directed mutagenesis.

Also within the scope of the invention are chimeric and humanized antibodies in which specific amino acids have been substituted, deleted or added. In particular, preferred humanized antibodies have amino acid substitutions in the framework region, such as to improve binding to the antigen. For example, in a humanized antibody having mouse CDRs, amino acids located in the human framework region can be replaced with the amino acids located at the corresponding positions in the mouse antibody. Such substitutions are known to improve binding of humanized antibodies to the antigen in some instances. Antibodies in which amino acids have been added, deleted, or substituted are referred to herein as modified antibodies or altered antibodies.

Pharmaceutical Compositions and Administration

The agents of the invention can be incorporated into compositions, e.g., pharmaceutical compositions, suitable for administration to a subject.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, i.e., an agent

described herein may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans with other antibodies. The preferred mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody is administered by intravenous infusion or injection. In another preferred embodiment, the antibody is administered by intramuscular or subcutaneous injection.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular,

subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, antibody or antibody portion) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

The compositions of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, *e.g.*, *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

In certain embodiments, compositions of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4.,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. These patents are incorporated herein by reference. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, the compounds of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, e.g., U.S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or

more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, e.g., V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685).

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an agent of the invention is 0.1-20 mg/kg, more preferably 1-10 mg/kg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an agent of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the agent may vary according to factors such as the disease state, age, sex, and weight of the individual,

and the ability of the antibody or antibody portion to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the agent is outweighed by the therapeutically beneficial effects. A "therapeutically effective dosage" preferably inhibits a measurable parameter, e.g., tumor growth by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The ability of a compound to inhibit a measurable parameter, e.g., cancer, can be evaluated in an animal model system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit, such inhibition *in vitro* by assays known to the skilled practitioner.

A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Combination Therapy

In one embodiment, the compositions of the invention, e.g., the pharmaceutical compositions, are administered in combination therapy, i.e., combined with other agents, e.g., therapeutic agents, that are useful for treating disorders, such as cancer or T cell- mediated disorders. The term "in combination" in this context means that the agents are given substantially contemporaneously, either simultaneously or sequentially. If given sequentially, at the onset of administration of the second compound, the first of the two compounds is preferably still detectable at effective concentrations at the site of treatment. For example, the combination therapy can include a composition of the present invention coformulated with, and/or coadministered with, one or more additional therapeutic agents, e.g., one or more anti-cancer agents, cytotoxic or cytostatic agents and/or immunosuppressants. For example, the agents of the invention or antibody binding fragments thereof may be coformulated with, and/or coadministered with, one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface

molecules), one or more cytokines, or immunosuppressants, e.g., cyclosporin A or FK506. Furthermore, one or more antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies.

The terms "cytotoxic agent" and "cytostatic agent" and "anti-tumor agent" are used interchangeably herein and refer to agents that have the property of inhibiting the growth or proliferation (e.g., a cytostatic agent), or inducing the killing, of hyperproliferative cells, e.g., an aberrant cancer cell or a T cell. In cancer therapeutic embodiment, the term "cytotoxic agent" is used interchangeably with the terms "anti-cancer" or "anti-tumor" to mean an agent, which inhibits the development or progression of a neoplasm, particularly a solid tumor, a soft tissue tumor, or a metastatic lesion.

Nonlimiting examples of anti-cancer agents include, e.g., antimicrotubule agents, topoisomerase inhibitors, antimetabolites, mitotic inhibitors, alkylating agents, intercalating agents, agents capable of interfering with a signal transduction pathway, agents that promotes apoptosis and radiation. Examples of the particular classes of anti-cancer agents are provided in detail as follows: antitubulin/antimicrotubule, e.g., paclitaxel, vincristine, vinblastine, vindesine, vinorelbin, taxotere; topoisomerase I inhibitors, e.g., topotecan, camptothecin, doxorubicin, etoposide, mitoxantrone, daunorubicin, idarubicin, teniposide, amsacrine, epirubicin, merbarone, piroxantrone hydrochloride; antimetabolites, e.g., 5-fluorouracil (5-FU), methotrexate, 6-mercaptopurine, 6-thioguanine, fludarabine phosphate, cytarabine/Ara-C, trimetrexate, gemcitabine, acivicin, alanosine, pyrazofurin, N-Phosphoracetyl-L-Asparate=PALA, pentostatin, 5-azacitidine, 5-Aza 2'-deoxycytidine, ara-A, cladribine, 5 - fluorouridine, FUDR, tiazofurin, N-[5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl]-L-glutamic acid; alkylating agents, e.g., cisplatin, carboplatin, mitomycin C, BCNU=Carmustine, melphalan, thiotepa, busulfan, chlorambucil, plicamycin, dacarbazine, ifosfamide phosphate, cyclophosphamide, nitrogen mustard, uracil mustard, pipobroman, 4-ipomeanol; agents acting via other mechanisms of action, e.g., dihydrolenperone, spiromustine,

and desipeptide; biological response modifiers, e.g., to enhance anti-tumor responses, such as interferon; apoptotic agents, such as actinomycin D; and anti-hormones, for example anti-estrogens such as tamoxifen or, for example antiandrogens such as 4'-cyano-3-(4-fluorophenylsulphonyl)-2-hydroxy-2-methyl-3'-(trifluoromethyl) propionanilide.

Particular combination of cytotoxic agents can be used depending on the condition to be treated. For example, when treating leukemias, in addition to radiation, the following drugs, usually in combinations with each other, are often used: vincristine, prednisone, methotrexate, mercaptopurine, cyclophosphamide, and cytarabine. In chronic leukemia, for example, busulfan, melphalan, and chlorambucil can be used in combination. All of the conventional anti-cancer drugs are highly toxic and tend to make patients quite ill while undergoing treatment. Vigorous therapy is based on the premise that unless every leukemic cell is destroyed, the residual cells will multiply and cause a relapse.

The agents of the invention can be used in combination with other therapeutic agents that inhibit the activity of immune cells to be used to treat immune or hematopoietic cell disorders or conditions, including, but not limited to, transplant rejection, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, among others. The use of the antibodies, or antibody fragments, of the invention in combination with other therapeutic agents is discussed in further detail below.

Accordingly, an agent of the invention can be used in combination with one or more antibodies directed at other targets involved in regulating immune responses, e.g., transplant rejection or graft-v-host disease. Non-limiting examples of agents for treating or preventing immune responses with which an agent, of the invention can be combined include the following: antibodies against cell surface molecules, including but not limited to CD25 (interleukin-2 receptor-α), CD11a (LFA-1), CD54 (ICAM-1), CD4, CD45, CD28/CTLA4, CD80 (B7-1) and/or CD86 (B7-2). In yet another embodiment, an agent of the invention is used in combination with one or more general immunosuppressive agents, such as cyclosporin A or FK506.

Nonlimiting examples of agents for treating or preventing rheumatoid arthritis with which an agent of the invention can be combined include the following: non-steroidal anti-inflammatory drug(s) (NSAIDs); cytokine suppressive anti-

inflammatory drug(s) (CSAIDs); CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see *e.g.*, *Arthritis & Rheumatism* (1995) Vol. 38, S185); DAB 486-IL-2 and/or DAB 389-IL-2 (IL-2 fusion proteins; Seragen; see *e.g.*, *Arthritis & Rheumatism* (1993) Vol. 36, 1223); Anti-Tac (humanized anti-IL-2R; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (*e.g.*, agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Amgen); TNF-bp/s-TNFR (soluble TNF binding protein; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284; *Amer. J. Physiol. - Heart and Circulatory Physiology* (1995) Vol. 268, pp. 37-42); R973401 (phosphodiesterase Type IV inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); MK-966 (COX-2 Inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S81); Iloprost (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S82); methotrexate; thalidomide (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282) and thalidomide-related drugs (*e.g.*, Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S131; *Inflammation Research* (1996) Vol. 45, pp. 103-107); tranexamic acid (inhibitor of plasminogen activation; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284); T-614 (cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); prostaglandin E1 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); Tenidap (non-steroidal anti-inflammatory drug; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S280); Naproxen (non-steroidal anti-inflammatory drug; see *e.g.*, *Neuro Report* (1996) Vol. 7, pp. 1209-1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-

inflammatory drug); Sulfasalazine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); Azathioprine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); ICE inhibitor (inhibitor of the enzyme interleukin-1□ converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70 or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitos of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (*e.g.*, SB203580); TNF-convertase inhibitors; anti-IL-12 antibodies; interleukin-11 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S296); interleukin-13 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S308); interleukin-17 inhibitors (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S120); gold; penicillamine; chloroquine; hydroxychloroquine; chlorambucil; cyclophosphamide; cyclosporine; total lymphoid irradiation; anti-thymocyte globulin; anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline; anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see *e.g.*, DeLuca et al. (1995) *Rheum. Dis. Clin. North Am.* 21:759-777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amiprilose (therafectin); cladribine (2-chlorodeoxyadenosine); and azaribine.

Nonlimiting examples of agents for treating or preventing inflammatory bowel disease with which an agent of the invention can be combined include the following: budenoside; epidermal growth factor; corticosteroids; cyclosporin, sulfasalazine; aminosalicylates; 6-mercaptopurine; azathioprine; metronidazole; lipoxygenase inhibitors; mesalamine; olsalazine; balsalazide; antioxidants; thromboxane inhibitors; IL-1 receptor antagonists; anti-IL-1 monoclonal antibodies; anti-IL-6 monoclonal antibodies; growth factors; elastase inhibitors; pyridinyl-imidazole compounds; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric

anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); interleukin-10 (SCH 52000; Schering Plough); IL-4; IL-10 and/or IL-4 agonists (*e.g.*, agonist antibodies); interleukin-11; glucuronide- or dextran-conjugated prodrugs of prednisolone, dexamethasone or budesonide; ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); slow-release mesalazine; methotrexate; antagonists of Platelet Activating Factor (PAF); ciprofloxacin; and lignocaine.

Nonlimiting examples of agents for treating or preventing multiple sclerosis with which an agent of the invention can be combined include the following: corticosteroids; prednisolone; methylprednisolone; azathioprine; cyclophosphamide; cyclosporine; methotrexate; 4-aminopyridine; tizanidine; interferon-α1a (Avonex™; Biogen); interferon-1b (Betaseron™; Chiron/Berlex); Copolymer 1 (Cop-1; Copaxone™; Teva Pharmaceutical Industries, Inc.); hyperbaric oxygen; intravenous immunoglobulin; clabribine; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IL-10; IL-4; and IL-10 and/or IL-4 agonists (*e.g.*, agonist antibodies).

Another aspect of the present invention accordingly relates to kits for carrying out the combined administration of the agents with other therapeutic compounds. In one embodiment, the kit comprises an agent formulated in a pharmaceutical carrier, and at least one cytotoxic agent, formulated as appropriate, in one or more separate pharmaceutical preparations.

Uses of the Invention

The molecules of the invention, e.g., agents, described herein have therapeutic utilities. For example, these agents can be administered to cells in culture, e.g. *in vitro* or *ex vivo*, or in a subject, e.g., *in vivo*, to treat or diagnose a variety of disorders.

As used herein, the term "subject" is intended to include human and non-human animals. Preferred human animals include a human patient suffering from an unwanted immune response. Other preferred human animals include a cancer patient, or a subject in need of heightened immune surveillance, e.g., a patient suffering from a other or a subject suffering from a pathogenic infection, e.g., a viral, bacterial, or parasitic infection. The term "non-human animals" of the invention includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc.

The agents of the invention can be used to treat, and/or prevent disorders, such as cancers, immune cell disorders, e.g., T cell disorders, and infectious disorders.

As used herein, the terms "cancer", "hyperproliferative", "malignant", and "neoplastic" are used interchangeably, and refer to those cells an abnormal state or condition characterized by rapid proliferation or neoplasm. The terms are meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. "Pathologic hyperproliferative" cells occur in disease states characterized by malignant tumor growth.

The common medical meaning of the term "neoplasia" refers to "new cell growth" that results as a loss of responsiveness to normal growth controls, e.g. to neoplastic cell growth. A "hyperplasia" refers to cells undergoing an abnormally high rate of growth.

However, as used herein, the terms neoplasia and hyperplasia can be used interchangeably, as their context will reveal, referring generally to cells experiencing abnormal cell growth rates. Neoplasias and hyperplasias include "tumors," which may be either benign, premalignant or malignant.

The subject method can be useful in treating malignancies of the various organ systems, such as those affecting lung, breast, lymphoid, gastrointestinal (e.g., colon), and genitourinary tract (e.g., prostate), pharynx, as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus. Exemplary solid tumors that can be treated include: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma,

osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

The term "carcinoma" is recognized by those skilled in the art and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, e.g., which include malignant tumors composed of carcinomatous and sarcomatous tissues. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures.

The term "sarcoma" is recognized by those skilled in the art and refers to malignant tumors of mesenchymal derivation.

The subject method can also be used to inhibit the proliferation of hyperplastic/neoplastic cells of hematopoietic origin, e.g., arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. For instance, the present invention contemplates the treatment of various myeloid disorders including, but not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus, L. (1991) *Crit Rev. in Oncol./Hemotol.* 11:267-97). Lymphoid malignancies which may be treated by the

subject method include, but are not limited to acute lymphoblastic leukemia (ALL), which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas contemplated by the treatment method of the present invention include, but are not limited to, non-Hodgkin's lymphoma and variants thereof, peripheral T-cell lymphomas, adult T-cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF) and Hodgkin's disease.

As used herein, the terms "leukemia" or "leukemic cancer" refers to all cancers or neoplasias of the hematopoietic and immune systems (blood and lymphatic system). These terms refer to a progressive, malignant disease of the blood-forming organs, marked by distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow. The acute and chronic leukemias, together with the other types of tumors of the blood, bone marrow cells (myelomas), and lymph tissue (lymphomas), cause about 10% of all cancer deaths and about 50% of all cancer deaths in children and adults less than 30 years old. Chronic myelogenous leukemia (CML), also known as chronic granulocytic leukemia (CGL), is a neoplastic disorder of the hematopoietic stem cell.

The subject method can also be used to modulate (e.g., inhibit) the activity, e.g., proliferation, differentiation, survival) of an immune or hematopoietic cell (e.g., a cell of myeloid, lymphoid, erythroid lineages, or precursor cells thereof), and, thus, can be used to treat or prevent a variety of immune disorders. Non-limiting examples of the disorders that can be treated or prevented include, but are not limited to, transplant rejection, autoimmune diseases (including, for example, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy,

idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue. lichen planus, Graves' disease, sarcoidosis, primary biliary cirrhosis, uveitis posterior. and interstitial lung fibrosis), graft-versus-host disease, and allergy such as. atopic allergy.

Methods of Identifying Anergy Associated Nucleic Acids

In another aspect, the invention features, a method of analyzing NFAT-mediated gene expression. This method can be used to identify nucleic acids having an expression which is modulated by imbalanced activation of NFAT. relative to the NFAT-NFAT ligand or NFAT ligand alone. Such nucleic acids are candidates for inducing, maintaining, and/or propagating an anergic or tolerant state (also referred to herein as "anergy-associated nucleic acids").

In one embodiment, nucleic acids (e.g., RNAs or cDNAs) from the cell are analyzed by one or more of: DNA arrays, subtractive strategies, or genome-wide retroviral insertion.

In a preferred embodiment, nucleic acids (e.g., RNAs or cDNAs) from a cell is contacted with an array having a plurality of probes, wherein each of said probes of the plurality is positionally distinguished from the other probes in the plurality, thereby identifying nucleic acids which are modulated by modulation of an NFAT-NFAT ligand interaction.

DNA array technology can be used to identify target nucleic acids that are differentially expressed in ionomycin-stimulated T cells that are or are not expressing VIVIT (please see Example 11 for a description of VIVIT), to identify NFAT-dependent nucleic acids that are turned on in anergic T cells. Fully-stimulated T cells (PMA+ionomycin, anti-CD3+anti-CD28 or antigen/APC) expressing or not expressing VIVIT, can also be examined to identify NFAT-dependent nucleic acids that are turned on under conditions of full stimulation. The difference between these results will identify NFAT-dependent nucleic acids that are turned on in the presence versus absence of AP-1, NFκB, etc

DNA array experiments are preferably done with human T cells since available arrays and expressed sequence tag (EST) databases are highly biased

towards human genes. Therefore, available human T cell lines can be tested for their ability to be anergized under the conditions described in the Examples herein. Methods for stably expressing GFP-VIVIT or preferably, ER-VIVIT at high levels in these cell lines using the high-copy-number EBV-based episomal vectors mentioned can be developed.

Once a human T cell line stably expressing GFP-VIVIT or ER-VIVIT has been obtained and optimal conditions for anergizing human T cells have been established, RNA from 6h or 16 h ionomycin-stimulated or fully-stimulated T cells expressing or not expressing GFP-VIVIT (or treated or not treated with tamoxifen for cells expressing ER-VIVIT) can be made, to identify NFAT-dependent nucleic acids that are turned on under anergizing or fully stimulatory conditions. Array technology can be used to identify the nucleic acids expressed under these four conditions. RNA can be made by the FastTrack (Invitrogen) method; RNA from about 5 x 10^8 cells suffices for several experiments.

Subtractive strategies (RDA) provide another approach, attractive because it has a different bias from the DNA array approach. A modification of representational difference analysis (RDA) will be used to identify the nucleic acids that differ in ionomycin-stimulated T cells expressing or not expressing VIVIT.

Genome-wide retroviral insertion strategy (Wurst W et al. (1995) *Genetics* 139: 889-899; Forrester LM et al. (1996) Proc Natl Acad Sci USA 93: 1677-1682; Gogos JA et al. (1997) J Virol 71: 1644-1650; Whitney M et al. Nat Biotechnol 16: 1329-1333; Zambrowicz BP et al. (1998) Nature 392: 608-611) can also be used to select for cells that have integrated a retroviral vector randomly into a gene necessary for maintaining the anergic state, and so are able to escape from the proliferative block observed in ionomycin-stimulated cells that are reexposed to a full antigenic stimulus.

In addition to the methods described above, candidate nucleic acids that can be evaluated for their involvement in induction and maintenance of anergy include: the inhibitory cell surface receptor CTLA-4; the immunosuppressive cytokines IL-10 and TGFa; the RING finger E3 ubiquitin ligase Cbl; the tyrosine phosphatase SHP-2 that is thought to be recruited to the TCR via its binding to CTLA-4; and the small G protein and Ras antagonist Rap1. Each one of these candidates can be evaluated for

whether their protein or mRNA levels are elevated in cells exposed to tolerising stimuli (ionomycin or plate-bound anti-CD3); if so, whether the increase is inhibited by CsA, which inhibits tolerance induction; whether the increase is less pronounced in NFAT1-/- T cells, which are less capable of being tolerised; and whether the increase is inhibited by the selective NFAT inhibitor VIVIT.

Nucleic acids that are identified in more than one of the screens described above, or whose products have clear characteristics of (negative) signalling proteins, will be the first candidates to take through further analysis. The criteria for gene selection is as follows. (i) whether the mRNA (and if possible, the protein products) of the selected nucleic acids are expressed at significantly higher levels in anergic T cells than in resting or productively stimulated T cells; if so, whether the increase is inhibited by CsA, which inhibits tolerance induction; whether the increase is less pronounced in NFAT1-/- T cells, which are less capable of being tolerised; and whether the increase is inhibited by the selective NFAT inhibitor VIVIT. (ii) If so, full length cDNAs for the putative anergy-inducing nucleic acids will be obtained and tested to determine whether their overexpression renders T cells unresponsive to full TCR + CD28 stimulation, but not to stimulation with PMA + ionomycin which bypasses membrane-proximal signalling events. (iii) the final criteria is whether overexpression interferes with TCR-Ras coupling and the other proximal signalling events.

Nucleic Acids, Vectors and Host Cells

Another aspect of the invention pertains to isolated nucleic acid, vector and host cell compositions that can be used for expression of the NFAT, NFAT ligand, and/or anergy associated nucleic acids of the invention.

Nucleic acids of the inventor can be chosen for having codons, which are preferred, or non preferred, for a particular expression system. E.g., the nucleic acid can be one in which at least one codon, at preferably at least 10%, or 20% of the codons has been altered such that the sequence is optimized for expression in *E. coli*, yeast, human, insect, or CHO cells.

In a preferred embodiment, the nucleic acid differs (e.g., differs by substitution, insertion, or deletion) from that of the sequences provided, e.g., as follows: by at least

one but less than 10, 20, 30, or 40 nucleotides; at least one but less than 1%, 5%, 10% or 20% of the nucleotides in the subject nucleic acid. If necessary for this analysis the sequences should be aligned for maximum homology. "Looped" out sequences from deletions or insertions, or mismatches, are considered differences. The differences are, preferably, differences or changes at nucleotides encoding a non-essential residue(s) or a conservative substitution(s).

The terms "host cell" and "recombinant host cell" are used interchangeably herein. Such terms refer not only to the particular subject cell, but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein. A host cell can be any prokaryotic, e.g., bacterial cells such as *E. coli*, or eukaryotic, e.g., insect cells, yeast, or preferably mammalian cells (e.g., cultured cell or a cell line). Other suitable host cells are known to those skilled in the art.

Preferred mammalian host cells for expressing the anergy-associated nucleic acids of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in R.J. Kaufman and P.A. Sharp (1982) *Mol. Biol.* 159:601-621), lymphocytic cell lines, e.g., NS0 myeloma cells and SP2 cells, COS cells, and a cell from a transgenic animal, e.g., e.g., mammary epithelial cell.

In another aspect, the invention features a vector, e.g., a recombinant expression vector. The recombinant expression vectors of the invention can be designed for expression of the anergy-associated nucleic acids, in prokaryotic or eukaryotic cells. For example, polypeptides of the invention can be expressed in *E. coli*, insect cells (e.g., using baculovirus expression vectors), yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, (1990) *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA. Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins.

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that are operatively linked and control the expression of the antibody chain genes in a host cell.

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. With respect to transcription regulatory sequences, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. For switch sequences, operably linked indicates that the sequences are capable of effecting switch recombination.

The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner *et al.*

In addition to the nucleic acids and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see e.g., U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr$^-$ host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

Standard recombinant DNA methodologies are used obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), Ausubel, F.M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989).

Nucleic Acid Arrays

Arrays are useful molecular tools for characterizing a sample by multiple criteria. For example, an array having a capture probes for one or more anergy-associated nucleic acids can be used to assess the anergic state of an immune cell.

Arrays can be fabricated by a variety of methods, e.g., photolithographic methods (see, e.g., U.S. Patent Nos. 5,143,854; 5,510,270; and. 5,527,681), mechanical methods (e.g., directed-flow methods as described in U.S. Patent No. 5,384,261), pin based methods (e.g., as described in U.S. Pat. No. 5,288,514), and bead based techniques (e.g., as described in PCT US/93/04145). The capture probe can be a single-stranded nucleic acid, a double-stranded nucleic acid (e.g., which is denatured during hybridization), or a nucleic acid having a single-stranded region and a double-stranded region. Preferably, the capture probe is single-stranded.

The isolated nucleic acid is preferably mRNA that can be isolated by routine methods, e.g., including DNase treatment to remove genomic DNA and hybridization to an oligo-dT coupled solid substrate (e.g., as described in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y).

Optionally, the isolated mRNA can be reversed transcribed and optionally amplified, e.g., by rtPCR, e.g., as described in (Mullis (1987) U.S. Patent No. 4,683,202). The nucleic acid can be an amplification product, e.g., from PCR (U.S. Patent No. 4,683,196 and 4,683,202); rolling circle amplification ("RCA," U.S. Patent No. 5,714,320), isothermal RNA amplification or NASBA (U.S. Patent Nos. 5,130,238; 5,409,818; and 5,554,517), and strand displacement amplification (U.S. Patent No. 5,455,166). The nucleic acid can be labeled during amplification, e.g., by the incorporation of a labeled nucleotide. Examples of preferred labels include fluorescent labels, e.g., red-fluorescent dye Cy5 (Amersham) or green-fluorescent dye Cy3 (Amersham), and chemiluminescent labels, e.g., as described in U.S. Patent No. 4,277,437. Alternatively, the nucleic acid can be labeled with biotin, and detected after hybridization with labeled streptavidin, e.g., streptavidin-phycoerythrin (Molecular Probes).

The labeled nucleic acid can be contacted to the array. In addition, a control nucleic acid or a reference nucleic acid can be contacted to the same array. The

control nucleic acid or reference nucleic acid can be labeled with a label other than the sample nucleic acid, e.g., one with a different emission maxima. Labeled nucleic acids can be contacted to an array under hybridization conditions. The array can be washed, and then imaged to detect fluorescence at each address of the array.

Nucleic acids which are similarly regulated during a change in T cell state, e.g., a change in NFAT activity can be identified by clustering expression data to identify coregulated nucleic acids. Nucleic acids can be clustered using hierarchical clustering (see, e.g., Sokal and Michener (1958) *Univ. Kans. Sci. Bull.* 38:1409), Bayesian clustering, k-means clustering, and self-organizing maps (see, Tamayo *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:2907).

Expression profiles obtained from gene expression analysis on an array can be used to compare samples and/or cells in a variety of states as described in Golub *et al.* ((1999) *Science* 286:531). In one embodiment, multiple expression profiles from different conditions and including replicates or like samples from similar conditions are compared to identify nucleic acids whose expression level is predictive of the sample and/or condition. Each candidate gene can be given a weighted "voting" factor dependent on the degree of correlation of the gene's expression and the sample identity. A correlation can be measured using a Euclidean distance or the Pearson correlation coefficient.

The similarity of a sample expression profile to a predictor expression profile (e.g., a reference expression profile that has associated weighting factors for each gene) can then be determined, e.g., by comparing the log of the expression level of the sample to the log of the predictor or reference expression value and adjusting the comparison by the weighting factor for all nucleic acids of predictive value in the profile.

Polypeptide Arrays

A low-density (96 well format) protein array has been developed in which proteins are spotted onto a nitrocellulose membrane Ge, H. (2000) *Nucleic Acids Res.* *28*, e3, I-VII). A high-density protein array (100,000 samples within 222 X 222 mm) used for antibody screening was formed by spotting proteins onto polyvinylidene difluoride (PVDF) (Lueking *et al.* (1999) *Anal. Biochem. 270*, 103-111).

Polypeptides can be printed on a flat glass plate that contained wells formed by an enclosing hydrophobic Teflon mask (Mendoza, *et al.* (1999). *Biotechniques 27*, 778-788.). Also, polypeptide can be covalently linked to chemically derivatized flat glass slides in a high-density array (1600 spots per square centimeter) (MacBeath, G., and Schreiber, S.L. (2000) *Science 289*, 1760-1763). De Wildt *et al.*, describe a high-density array of 18,342 bacterial clones, each expressing a different single-chain antibody, in order to screening antibody-antigen interactions (De Wildt *et al.* (2000). *Nature Biotech. 18*, 989-994). These art-known methods and other can be used to generate an array of antibodies for detecting the abundance of polypeptides in a sample. The sample can be labeled, e.g., biotinylated, for subsequent detection with streptavidin coupled to a fluorescent label. The array can then be scanned to measure binding at each address.

Reporter Gene Assays

In another implementation, a reporter gene is utilized to monitor the expression of an anergy-assoaciated. Such a reporter can be useful for high-throughput screens for agents which alter a T cell state.

To construct the reporter, the promoter of the selected gene can be operably linked to the reporter gene, e.g., without utilizing the reading frame of the selected gene. The nucleic acid construction is transformed into tissue culture cells, e.g., T cells, by a transfection protocol or lipofection to generate reporter cells.

In one embodiment, the reporter gene is green fluorescent protein. In a second implementation, the reporter is β-galactosidase. In still other embodiments, the reporter gene is alkaline phosphatase, β-lactamase, luciferase, or chloramphenicol acetyltransferase. The nucleic acid construction can be maintained on an episome or inserted into a chromosome, for example using targeted homologous recombination as described in Chappel, US 5,272,071 and WO 91/06667.

In the implementation utilizing green fluorescent protein (GFP) or enhanced GFP (eGFP) (Clontech, Palo Alto, CA) the reporter cells are grown in microtiter plates wherein each well is contacted with a unique agent to be tested. Following a desired treatment duration, e.g., 5 hours, 10 hours, 20 hours, 40 hours, or 80 hours, the microtiter plate is scanned under a microscope using UV lamp emitting light at 488

nm. A CCD camera and filters set to detect light at 509 nm is used to monitor the fluorescence of eGFP, the detected fluorescence being proportional to the amount of reporter produced.

In the implementation utilizing β-galactosidase, a substrate which produces a luminescent product in a reaction catalyzed by β-galactosidase is used. Again reporter cells are grown in microtiter plates and contacted with compounds for testing. Following treatment, cells are lysed in the well using a detergent buffer and exposed to the substrate. Lysis and substrate addition is achieved in a single step by adding a buffer which contains a 1:40 dilution of Galacton-Star™ substrate (3-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(4'chloro)-tricyclo-[3.3.1.1^{3,7}] decan}-4-yl)phenyl-B-D-galactopyranoside; Tropix, Inc., Cat.# GS100), a 1:5 dilution of Sapphire II™ luminescence signal enhancer (Tropix, Inc., Cat.#LAX250), 0.03% sodium deoxycholic acid, 0.053% CTAB, 250 mM NaCl, 300 mM HEPES, pH 7.5). The cells are incubated in the mixture at room temperature for approximately 2 hours prior to quantitation. β-galactosidase activity is monitored by the chemiluminescence produced by the product of β-galactosidase hydrolysis of the Galacton-Star™ substrate. A microplate reader fitted with a sensor is used to quantitate the light signal. Standard software, for example, Spotfire Pro version 4.0 data analysis software, is utilized to analyze the results. The mean chemiluminescent signal for untreated cells is determined. Compounds which exhibit a signal at least 2.5 standard deviations above the mean are candidates for further analysis and testing. Similarly, for alkaline phosphatase, β-lactamase, and luciferase, substrates are available which are fluorescent when converted to product by enzyme.

The following invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

The Example described below support a role for NFAT in tolerance induction: (i) the tolerizing stimulus can be provided by using a calcium ionophore, e.g.,

ionomycin alone; (ii) NFAT1-negative T cells are less readily anergized than NFAT1+/+ T cells in culture; and (iii) anergy induction is blocked by CsA.

Example 1: NFAT in tolerance induction

Ionomycin pretreatment attenuates the proliferation of a Th1 clone to antigen. To test that NFAT plays a role in anergy induction in T cells, arsonate-I-Ad-specific murine Th1 T cell clone D5 (Ar-5; Rao A et al. (1984) Cell 36: 889-895) were used to set up the model of clonal anergy developed by Schwartz and coworkers (Schwartz RH. (1996) J Exp Med 184: 1-8). As the anergy-inducing stimulus, the calcium ionophore, ionomycin, was used which causes nuclear translocation and activation of NFAT proteins at concentrations that do not activate other signaling pathways or transcription factors, including MAP kinase pathways, AP-1, and NFkB. Pretreatment of D5 T cells with ionomycin greatly diminished their subsequent proliferative response to antigen (Figure 2) or anti-CD3. These doses of ionomycin did not elicit cell proliferation and had no effect on the viability of D5 cells. 16 h of ionomycin pretreatment sufficed for complete unresponsiveness in the subsequent response. For convenience in the following experiments, 16 hours of ionomycin pretreatment were used to induce unresponsiveness, and monitored anergy induction by measuring responses to antigen rather than anti-CD3.

Example 2: Ionomycin Pretreatment Attenuates Induced Transcription of the IL-2 and IFN-γ Nucleic acids

The effect of ionomycin pretreatment on inducible gene transcription in D5 T cells was examined by monitoring proliferation and transcript levels in parallel. D5 cells were cultured alone or with 200 nM ionomycin for 16 h, then washed and cultured with irradiated splenic APC in the presence or absence of antigen. 3H-thymidine incorporation was assessed at 48 h. Total cellular RNA was isolated at 6 h and analyzed by RNase protection assay for transcript levels of the indicated cytokines.

Under conditions where ionomycin pretreatment reduced the proliferative response to antigen by >95%, a complete loss of IL-2 transcripts and substantial reduction in IFN-γ, TNF-α, GM-CSF and MIP-1α transcripts were observed in

response to the second stimulation with antigen and APC. FasL transcript levels were not decreased, or were only slightly decreased, in ionomycin-pretreated cells, indicating that ionomycin pretreatment did not universally downregulate gene expression, and suggesting that Fas-mediated apoptosis was unlikely to be involved in ionomycin-mediated unresponsiveness.

Example 3: Recently-differentiated Th1 cells are susceptible to anergy induction

To study the effect of ionomycin pretreatment on the response of primary T cells to antigen. CD4+ T cells were purified from spleen and lymph nodes from DO11.10 TcR transgenic mice and differentiated in Th1 or Th2 directions as previously described (Kiani A et al. (1997) *Immunity* 7: 849-860; Agarwal S. et al. (1998) *Immunity* 9: 765-775). CD4 T cells from spleen and lymph nodes of DO11.10 TCR-transgenic mice were differentiated for 6 days in the presence of irradiated antigen-pulsed splenic APC under Th1 or Th2 conditions. Cells were washed, pretreated with 200 nM ionomycin for 16 hours, washed, and restimulated for 6 h with irradiated splenic APC in the presence or absence of antigen. Total cellular RNA was isolated and analysed by RNase protection assay for transcript levels of the indicated cytokines.

Ionomycin pretreatment of differentiated Th1 cells led to a marked reduction in their ability to produce IL-2, IFN-γ, IL-10, TNF-α and MIP-1α mRNAs upon subsequent antigen stimulation; in particular, IL-2 transcripts were undetectable. As shown above for D5 T cells, ionomycin pretreatment of recently differentiated Th1 cells from DO11.10 TCR transgenic mice did not alter their levels of Fas-L transcripts. Ionomycin pretreatment was less efficient in causing unresponsiveness in recently differentiated Th2 cells from DO11.10 TCR transgenic mice, although ~50-70% reduction in transcript levels was observed for IL-4, IL-5 and IL-13. Interestingly, IL-10 transcript levels were not affected by ionomycin pretreatment of Th2 cells, although they were greatly diminished by ionomycin pretreatment of Th1 cells.

Example 4: NFAT1-/- T cells are less readily anergized than wildtype T cells following ionomycin pretreatment

Since pretreatment with the low concentrations of ionomycin used in our experiments leads primarily to NFAT activation, the involvement of NFAT proteins in anergy induction was examined. To do this, we took advantage of the fact that the NFAT family member NFAT1 constitutes 80-90% of total NFAT in resting murine T cells. Targeted disruption of the NFAT1 gene in mice does not result in any compensatory increase in the levels of the other NFAT family members; thus cells from homozygous NFAT1-deficient mice not only lack all NFAT1, but also contain only ~10-15% the normal level of total NFAT (Xanthoudakis S. et al. (1996) Science 272: 892-895).

To test whether this profound deficiency in NFAT1 and total NFAT activity would affect the susceptibility of T cells to anergy induction in vitro, DO11.10 TcR transgenics with NFAT1-/- mice were bred, and compared the ability of ionomycin pretreatment to cause antigen specific unresponsiveness in NFAT1+/+ DO11.10 TcR transgenic mice versus their NFAT1-/- counterparts. Differentiated Th1 T cells from NFAT1+/+ DO11.10 transgenic mice showed a marked decrease in transcription of IL2 or IFN-γ nucleic acids when pretreated with ionomycin. This decrease was much less pronounced in Th1 cells from NFAT1-/- mice. As a control, the response of the ionomycin-pretreated cells to PMA and ionomycin was tested, stimuli which together mimic TCR engagement while bypassing the initial membrane-proximal steps of signal transduction through the TCR.

Cells were ionomycin-pretreated (200 nM/ 16 h) and restimulated for 6 h with antigen/APC (lanes 1-4) or 10 nM PMA and 1 mM ionomycin. Cytokine mRNA levels were measured. The ability to be anergised (anergy index = fold decrease in IL-2 and IFN-γ mRNA expression in pretreated relative to untreated T cells (quantified by PhosphorImager) is shown in Figure 3. As shown for T cells that have been anergized by other means (Schwartz RH. (1996) *supra*), Th1 cells anergized by ionomycin pretreatment were fully responsive to combined stimulation with PMA and ionomycin. They are also responsive to IL-2, and inclusion of IL-2 during the pretreatment step prevents anergy induction.

To quantify the anergizing effect of ionomycin, and to better compare its effect on NFAT1-/- versus NFAT1+/+ cells, we defined a parameter that we termed "anergy index" (ratio of cytokine production by cells not exposed versus exposed to the first anergising stimulus (ionomycin in this case)). This quotient which measures the relative decrease in the response of anergized relative to non-anergized T cells, is a semi-quantitative measure of the efficiency of anergy induction whose utility will be apparent from the Methods section below. Thus, in the experiment of Figure 5, ionomycin pretreatment caused an 18-fold decrease in the stimulated levels of IL-2 transcripts in NFAT1+/+ Th1 cells, but only a 2.5-fold decrease in IL-2 transcripts in NFAT1-/- Th1 cells: that is, the anergy index was 18 for NFAT1+/+ T cells and 2.5 for NFAT1-/- T cells (Figure 5, left). In the same experiment, ionomycin pretreatment caused 12- and 2.5-fold decreases in IFN-g transcripts in NFAT1+/+ and NFAT1-/- Th1 cells, respectively (Figure 5, right). These results indicate that NFAT1-/- T cells are significantly less capable than NFAT1+/+ T cells of being anergized in culture.

Example 5: Impaired anergy induction in Th1 cells from NFAT1-/- mice in response to anti-CD3 pretreatment

The anergy induced by pretreatment with ionomycin may differ somewhat from the anergy induced by stimulation through the T cell receptor in the absence of costimulatory ligands. Ionomycin-induced anergy appears to be short-lived (several hours), while that induced by stimulation with immobilised anti-CD3 is significantly longer-lived (several days). In Figure 4, Th1 cell lines generated from CD4 T cells from NFAT1+/+ and NFAT1-/- DO11.10 TCR-transgenic mice. Two weeks after the last stimulation, cells were anergized by exposure to plate-bound anti-CD3, then washed, rested for 3 days, and stimulated with APC plus OVA peptide for 24 hours. Secreted IL-2 was measured by ELISA. The results show the mean+S.E. of three independent experiments. The numbers at the bottom are the mean values of the anergy index, defined as the fold reduction of IL-2 production observed in anti-CD3-pretreated (right bar) relative to untreated (left bar) T cells.

Figure 4 shows that NFAT1-/- Th1 cells were effectively insusceptible to anergy induced by anti-CD3. The average tolerization index for wildtype T cells was

8.1 in multiple experiments, but 0.7 for NFAT1-/- T cells (i.e. the response of the untreated cells was actually slightly lower than that of the anti-CD3-pretreated cells). Again these results suggest that NFAT plays a role in this model of anergy induction.

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Example 6: CsA blocks anergy induction in NFAT1+/+ Th1 cells.

Inclusion of CsA during pretreatment with immobilised anti-CD3 prevents the induction of anergy (Figure 5). A Th1 cell line similar to that described in Figure 6 was anergised by stimulation with plate-bound anti-CD3 in the presence or absence of 1mM CsA, then washed, rested for 3 days, and restimulated with APC plus OVA peptide for 24 hours. Secreted IL-2 was measured by ELISA. The results show the mean+S.E. of three independent experiments.

An NFAT1+/+ Th1 cell line was effectively anergised by exposure to immobilised anti-CD3 for 16 h since it produced barely any IL-2 in response to subsequent stimulation with antigen/APC (compare two left hatched bars); the anergy index (fold decrease in response) was 14.1. Pretreatment with CsA alone for 16 h did not significantly affect the magnitude of the subsequent response (compare first and third hatched bars), indicating that this inhibitor was efficiently washed out during the following 3 day rest period. The cells that had been pretreated with anti-CD3 in the presence of CsA showed a slight enhancement of their subsequent response to antigen/APC (two-fold over that observed with CsA pretreatment alone, compare third and fourth hatched bars), yielding an anergy index of 0.5. Again these results implicate NFAT or another calcineurin-sensitive mechanism in the process of anergy induction.

Example 7: Development of a retroviral vector for reintroducing NFAT1 into NFAT1-/- primary murine T cells

A retroviral transduction system for introducing wildtype and mutant NFAT into primary CD4 T cells from NFAT1-/- mice was developed (Figure 6). CD4 T cells from spleen and lymph nodes of NFAT1-/- mice were stimulated under Th1 conditions with APC plus OVA peptide. 24 hours following stimulation, the cells were infected with MSCV-based retroviruses encoding NFAT1, NFAT1-AP-1-less mutant and control GFP retrovirus as indicated. Three days after infection cells were analysed by FACS for GFP expression. Percentages of GFP-expressing cells are shown. The retroviral vectors used are shown above. NFAT1 cDNA was inserted upstream of an IRES-GFP cotranslation unit. Asterisks indicate the mutated residues in the DNA-binding domain of the NFAT1 AP-1-interaction mutant.

CD4 T cells are infected one day after stimulation with MSCV-based retroviruses containing an IRES-GFP cotranslation unit (Feske S. et al. (1996) Eur J Immunol 26: 2119-2126), and analysed 3 d later for GFP expression. As shown in Figure 6, 15-20% transduction efficiency has been achieved and up to 45% efficiency has been observed with the control GFP retrovirus in some experiments.

Example 8: Reintroduction of NFAT1 into NFAT1-/- T cells restores their ability to be anergised

Wild-type and mutant NFAT1 were reintroduced into CD4 T cells and tested for their ability to be anergised. An entire population of mixed GFP-positive and GFP-negative cells derived from those shown in Figure 6 was anergised by stimulation with plate-bound anti-CD3, then washed, rested and restimulated with antigen and APC. IL-2 expression was evaluated in both the GFP-positive (presumed NFAT1-positive) and GFP-negative (presumed NFAT1-negative) cells by intracellular cytokine staining. The results were consistent with the view that reintroduction of either wildtype or AP-1 mutant NFAT1 would restore the ability to be anergised (i.e. GFP-positive cells expressing wildtype or mutant NFAT1 showed less staining for intracellular IL-2 after exposure to the tolerising anti-CD3 stimulus, while the GFP-negative cells in the same population showed no decrease in staining). However the experiment needs to be repeated since the overall levels of IL-2 staining were low and some crucial controls were lost.

Example 9: Generation of an AP-1 interaction mutant of NFAT1

Based on information from the crystal structure of the NFAT1-Fos-Jun-DNA complex (Chen L. et al. (1998) Nature 392: 42-48), three amino acids of NFAT1 that make critical contacts with Fos and Jun (R466A, I467A, T533G) have been mutated. These residues are completely conserved in NFAT2, NFAT3 and NFAT4 (see Fig. 2 of ref. (Chen L. et al. (1998) Nature 392: 42-48). Similar DNA binding but impaired Fos-Jun interaction relative to WT NFAT1. Similar transactivation of k3-CAT and TNFa-promoter-Luc plasmids but impaired transactivation of NFAT-AP-1 and IL2-promoter-Luc reporter plasmids (Figure 7). The AP-1 interaction mutant of NFAT1 binds DNA as well as wildtype NFAT1, but cannot form cooperative complexes with

Fos/Jun or Jun/Jun dimers. It is unable to transactivate a reporter plasmid driven by the ARRE-2 composite NFAT-AP-1 site, but is unimpaired in its ability to transactivate a reporter plasmid driven by the k3 site of the TNFa promoter, an AP-1-independent kB-like site (Figure 9). Similarly, the mutant is strongly transactivating at the TNFa-promoter at which NFAT-AP-1 cooperation is not required, but is highly inefficient on the IL-2-promoter.

Example 10: Wildtype NFAT1, but not the AP-1 interaction mutant, promotes activation-induced cell death

One biological difference between wildtype and mutant NFAT1 is their effect on activation-induced cell death (Figure 8). The D5 Th1 clone was stably transfected with expression plasmids encoding GFP or NFAT1DReg (Garcia-Rodriguez C, Rao A. (1998) *J Exp Med* 187: 2031-2036), either wildtype or bearing the R466A, I467A, T533G mutations which impair the NFAT-AP-1 interaction. D5 clones stably expressing GFP, NFAT1DReg or its AP-1-less mutant were stimulated with plate bound anti-CD3 (1 mg/ml). After 72 hours, cells were collected, fixed with 70% ethanol, stained with propidium iodide and analysed for cell cycle by flow cytometry. The percentage of apoptotic cells was estimated based on the number in the subdiploid peak. A similar effect was observed in cells stimulated with PMA and ionomycin (not shown).

NFAT1DReg lacks most of the regulatory domain of NFAT1, is constitutively localised throughout the nucleus and cytoplasm, and is constitutively partially activated relative to wildtype NFAT1. When the stably-transfected cells were exposed to PMA and ionomycin or immobilised anti-CD3, cells expressing wildtype NFAT1DReg showed a significant increase in activation-induced cell death while cells expressing the AP-1 interaction mutant version showed no increase above the levels observed with GFP-expressing cells (Figure 8). We conclude that NFAT-AP-1 cooperation is necessary for activation-induced cell death; the target nucleic acids remain to be identified but may include FasL.

Example 11: VIVIT: a potent and selective inhibitor of NFAT

A major calcineurin-targetting motif on NFAT proteins has been identified (Aramburu J, et al. (1998) *Molecular Cell* 1: 627-637). This motif, which has the consensus sequence PxIxIT, is conserved in the N-terminal region of NFAT proteins and is required for their effective interaction with calcineurin. A peptide spanning the PxIxIT motif of NFAT1 (SPRIEIT) blocked the dephosphorylation of NFAT proteins by calcineurin, thereby blocking the nuclear translocation and transcriptional function of NFAT. This peptide did not affect calcineurin phosphatase activity or the ability of calcineurin to activate other transcription factors such as NFκB.

More recently, these studies have been extended to obtain a more potent but equally selective peptide inhibitor of the NFAT-calcineurin interaction (Aramburu J. et al. (1999) Science 285: 2129-2133). Using degenerate peptide libraries based on the PxIxIT motif, we identified a peptide (VIVIT) that inhibited the NFAT-calcineurin interaction at 30 to 50-fold lower concentrations than the original SPRIEIT peptide. When expressed as a GFP fusion protein in cells, the VIVIT peptide was almost as potent as CsA in its ability to block the activation of NFAT-dependent nucleic acids. However it was considerably more selective than CsA, which blocks all calcineurin-dependent pathways. Thus CsA blocked the activation of both NFAT and NFkB reporters, while VIVIT selectively blocked the activation of NFAT. Likewise, several nucleic acids (TNF-β, LT-β) induced by endogenous NFAT in Jurkat T cells were blocked by CsA but not by the VIVIT peptide, indicating that they are activated via calcineurin-dependent but not NFAT-dependent pathways.

By introducing the VIVIT sequence into NFAT1 in place of the original SPRIEIT sequence, we obtained a version of NFAT1 that was hyperresponsive to calcineurin and so was activated at far lower stimulus concentrations than wildtype NFAT1 (Aramburu J. et al. (1999) supra). Indeed, NFAT1[VIVIT] was already partially activated by the low basal levels of calcineurin activity present in resting cells.

The VIVIT peptide and the NFAT1[VIVIT] protein can be used to mediate tolerance induction in T cells.

Example 12: Expression of the GFP-VIVIT fusion protein under tetracycline control

Expression plasmids in which GFP and GFP-VIVIT are expressed under control of a 7x TetOP-CMV promoter, derived from the pUHD10.3 plasmid have been constructed (Prodeus AP et al. (1998) *Immunity* 9: 721-731). Jurkat cells stably expressing the "Tet-off" version of the tetracycline transactivator (tTA) were transiently transfected with CMV-TetOP-driven GFP and GFP-VIVIT expression plasmids as indicated. Doxycycline was added immediately after transfection, and protein expression was analysed by Western blotting 24 h later.

These plasmids drive strong, doxycycline-sensitive protein expression in Jurkat cells stably expressing the "Tet-off" version of the tetracycline transactivator (tTA), but not in conventional Jurkat cells. Doxycycline completely prevented expression of the CMV-TetOP-driven proteins, but not of the identical proteins expressed under control of a standard CMV promoter.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A method of modulating an NFAT-mediated immune response, in a subject, comprising:

administering to a subject an agent, which modulates one or more NFAT signaling activities, in an amount sufficient to modulate an NFAT-mediated immune response.

2. The method of claim 1, wherein said one or more NFAT signaling activities is selected from the group consisting of: (i) an interaction between NFAT and an NFAT ligand; (ii) the level of an NFAT-NFAT ligand complex; (iii) the activity or expression of NFAT or an NFAT ligand; (iv) the activity or expression of at least one component of NFAT signaling and the activity or expression of at least one component of NFAT ligand signaling.

3. The method of claim 1, wherein said agent induces anergy.

4. The method of claim 2, wherein said agent inhibits anergy.

5. The method of claim 3, wherein the subject is a patient suffering from an unwanted immune response.

6. The method of claim 4, wherein the subject is a cancer patient, or a subject in need of heightened immune surveillance.

7. The method of claim 1, wherein the NFAT protein is selected from the group consisting of: NFAT1, NFAT2, NFAT3, and NFAT4.

8. A method for increasing an NFAT-mediated immune response, in a cell, or tolerance in a subject, comprising:

contacting an immune cell, or administering to a subject, an agent which activates one or more NFAT signaling activities, in an amount sufficient to activate an NFAT-mediated immune response.

9. A method for decreasing an NFAT-mediated immune response, in a cell, or tolerance in a subject, comprising:

contacting an immune cell, or administering to a subject, an agent, which inhibits one or more NFAT signaling, in an amount sufficient to inhibit an NFAT-mediated immune response.

10. A method for modulating a protein-protein interaction between NFAT and an NFAT ligand, comprising: contacting the NFAT ligand, or NFAT, with an agent that modulates an interaction between NFAT ligand and NFAT, such that the protein-protein interaction between the NFAT ligand and the NFAT is modulated.

11. The method of claim 11, wherein the level of protein-protein interaction is detected by the formation of an NFAT-NFAT ligand complex.

12. A method of modulating the expression of one or more anergy-associated nucleic acids, comprising:

contacting an immune cell with an agent that modulates NFAT signaling;

allowing expression of the anergy-associated nucleic acids to occur;

detecting expression of said nucleic acids.

13. A method of inducing or promoting anergy, comprising

introducing into a cell a nucleic acid encoding a mutant NFAT polypeptide; and

maintaining the cell under conditions such that the NFAT mutant polypeptide is expressed, thereby reducing or inhibiting the formation of an NFAT-NFAT ligand complex, and inducing or promoting anergy.

14. A method of evaluating, or identifying, an agent for the ability to inhibit an interaction between NFAT-1 and an NFAT ligand, comprising:

providing a test agent, an NFAT protein or an NFAT ligand-binding fragment thereof, and an NFAT ligand or an NFAT-binding fragment thereof;

contacting said test agent, said NFAT protein or fragment thereof, and said NFAT ligand or fragment thereof, under conditions that allow an interaction between NFAT and the NFAT ligand to occur; and

determining whether said test agent modulates, e.g., inhibits, the interaction between said NFAT protein or fragment thereof, and said NFAT ligand or fragment thereof,

wherein a change, e.g., a decrease, in the level of binding between said NFAT protein or fragment thereof, and said NFAT ligand or fragment thereof, is indicative of inhibition of the interaction between NFAT an NFAT ligand.

15. The method of claim 14, further comprising: contacting said agent with a test cell, or a test animal, to evaluate the effect of the test agent on the interaction between NFAT and an NFAT ligand.

16. A method of evaluating, or identifying, an agent for the ability to modulate transcription of an anergy-associated nucleic acid, comprsing:

contacting an immune cell, or an NFAT-containing transcription complex, with a test agent; and

determining whether said test agent modulates transcription of at least one anergy-associated nucleic acid, wherein a change in the level of expression of said anergy-associated nucleic acid is indicative of a modulation of the expression of anergy-associated nucleic acids.

17. The method of claim 16, wherein the level of expression of the at least one anergy-associated nucleic acid is evaluated after stimulation of the immune cell with an antigen.

18. An agent identified by the method of claim 14.

19. An agent identified by the method of claim 16.

20. A method of analyzing NFAT-mediated gene expression, comprising:

providing nucleic acids, or gene products, expressed in an immune cell in which anergy has been induced; and

analyzing nucleic acids, or gene products, expressed in said cell, compared to the expression of the same nucleic acids, or gene products, in a cell in which anergy has not been induced, or in which immune activity has been modulated differently, thereby identifying nucleic acids or gene products which are modulated by NFAT imbalanced activation.

21. The method of claim 20, wherein anergy is induced by inhibiting the interaction between NFAT and an upstream NFAT activator.

22. The method of claim 21, wherein the nucleic acids from the cell are analyzed by one or more of: DNA arrays, subtractive strategies, and genome-wide retroviral insertion.

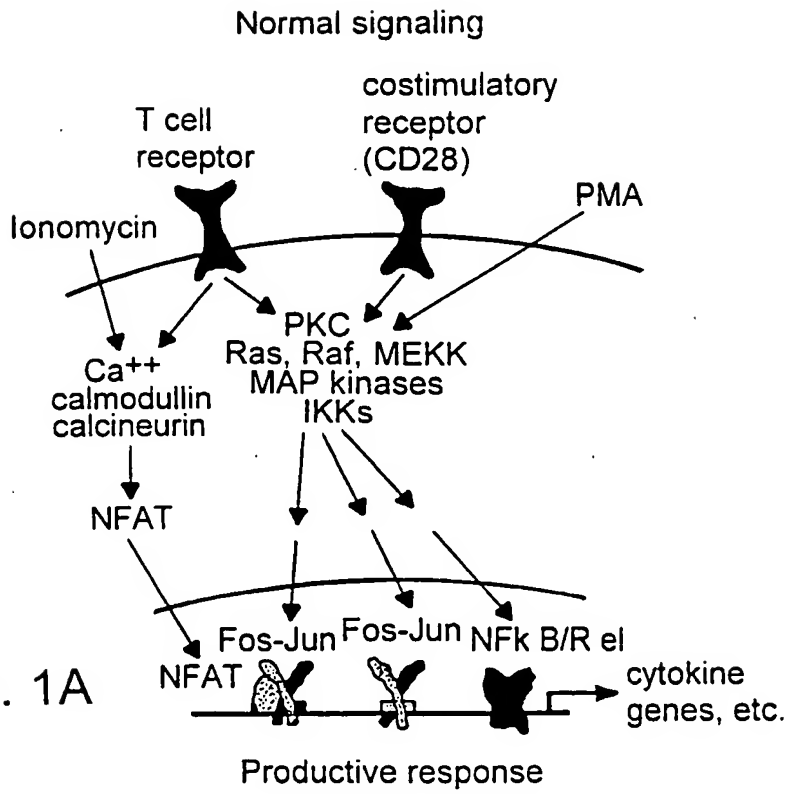
Normal signaling
costimulatory receptor (CD28)
T cell receptor
PMA
Ionomycin
PKC
Ras, Raf, MEKK
MAP kinases
IKKs
Ca++
calmodullin
calcineurin
NFAT
Fos-Jun
Fos-Jun
NFk B/R el
NFAT
cytokine genes, etc.
Productive response

FIG. 1A

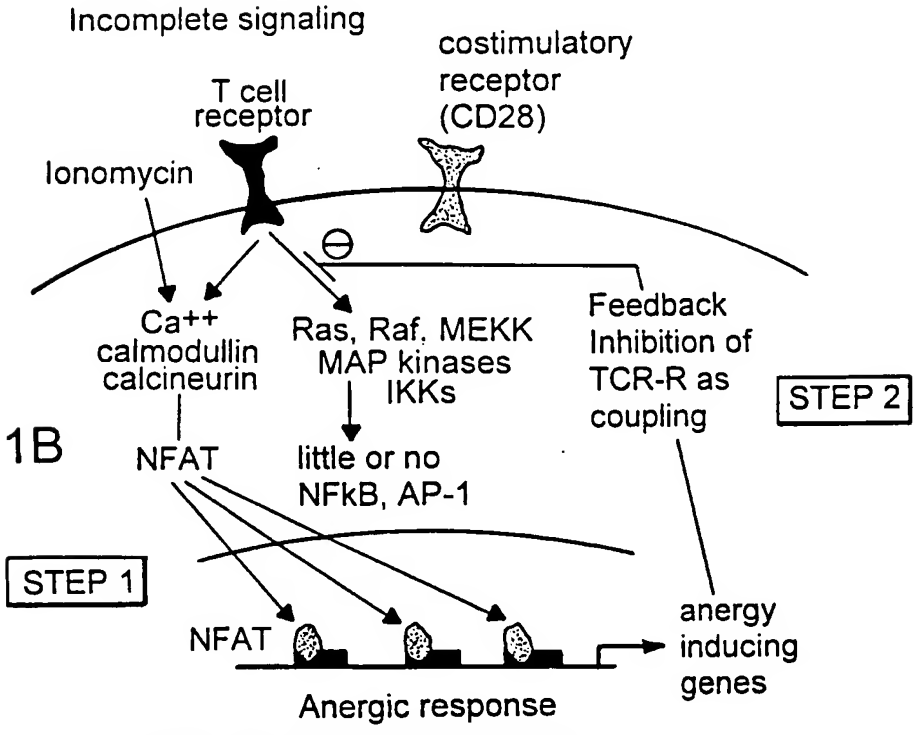
Incomplete signaling
costimulatory receptor (CD28)
T cell receptor
Ionomycin
Feedback Inhibition of TCR-R as coupling
STEP 2
Ca++
calmodullin
calcineurin
Ras, Raf, MEKK
MAP kinases
IKKs
NFAT
little or no NFkB, AP-1
STEP 1
NFAT
anergy inducing genes
Anergic response

FIG. 1B

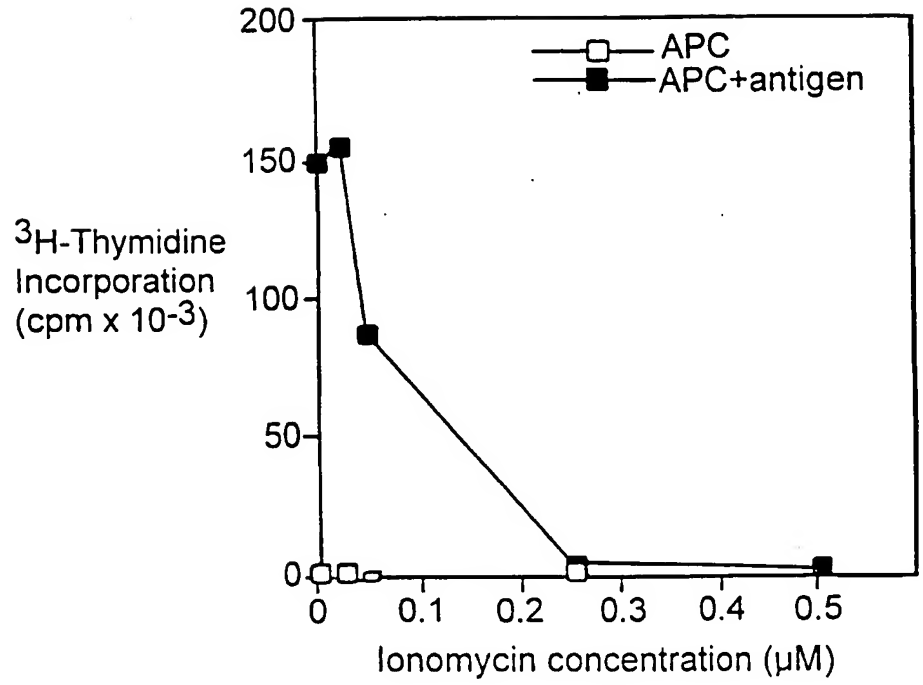
200
150
100
50
0
APC
APC+antigen
^{3}H-Thymidine
Incorporation
(cpm x 10^{-3})
0
0.1
0.2
0.3
0.4
0.5
Ionomycin concentration (μM)

FIG. 2

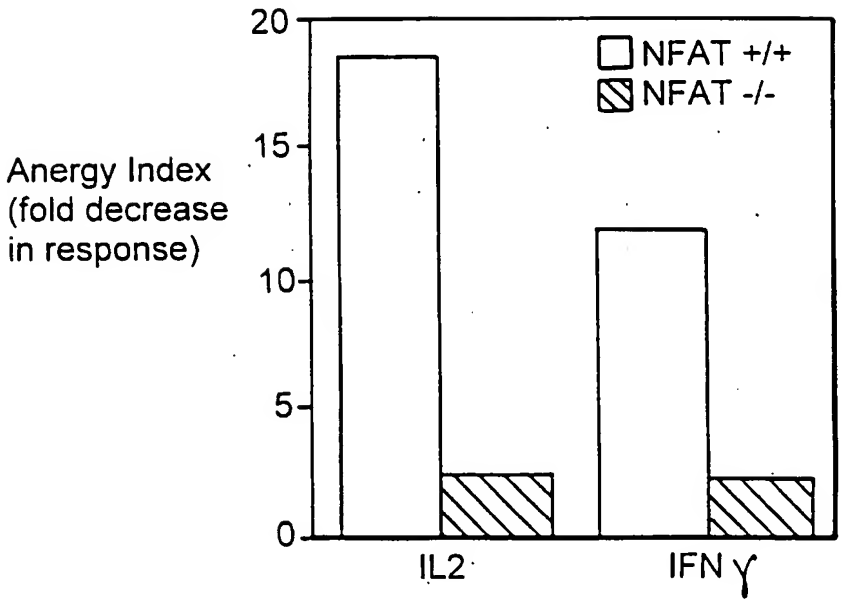
Anergy Index
(fold decrease
in response)
20
15
10
5
0
NFAT +/+
NFAT -/-
IL2
IFN γ

FIG. 3

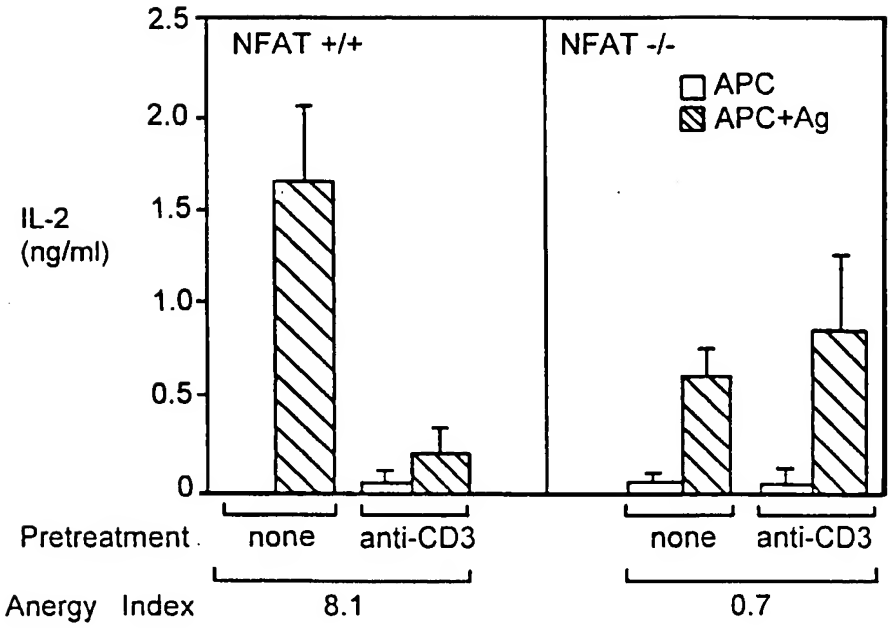
2.5
2.0
1.5
1.0
0.5
0
IL-2
(ng/ml)
NFAT +/+
NFAT -/-
APC
APC+Ag
Pretreatment
none
anti-CD3
none
anti-CD3
Anergy Index
8.1
0.7

FIG. 4

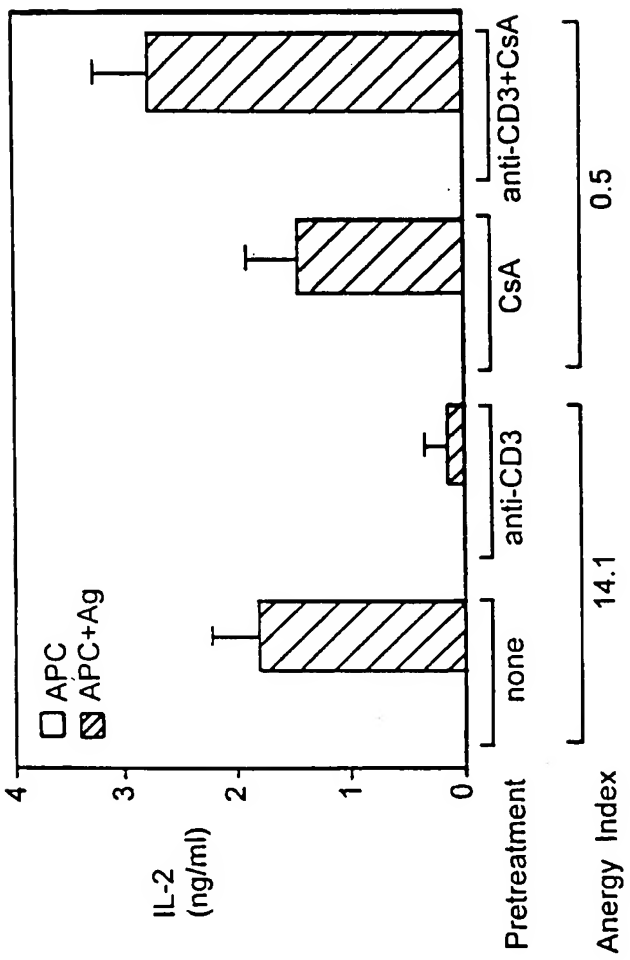
APC
APC+Ag
IL-2 (ng/ml)
0
1
2
3
4
Pretreatment
none
anti-CD3
CsA
anti-CD3+CsA
Anergy Index
14.1
0.5

FIG. 5

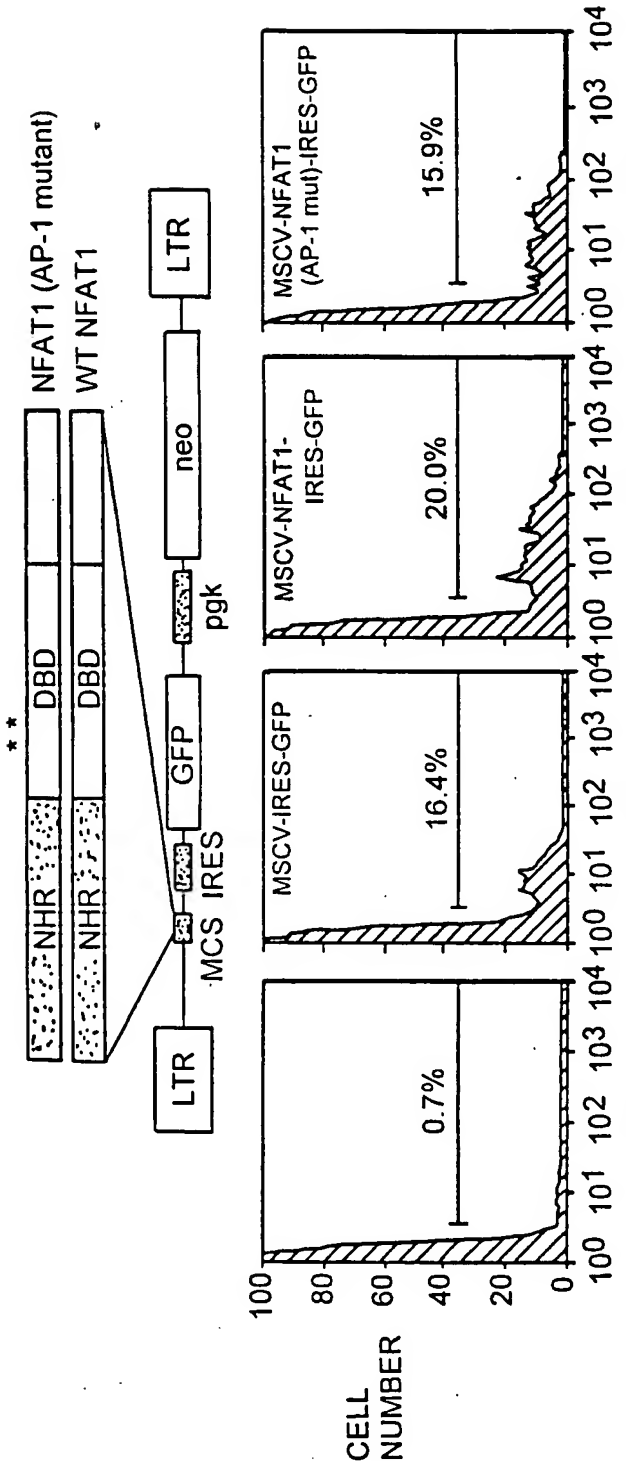
NFAT1 (AP-1 mutant)
WT NFAT1
NHR
DBD
LTR
MCS
IRES
GFP
pgk
neo
LTR
MSCV-IRES-GFP
0.7%
16.4%
MSCV-NFAT1-IRES-GFP
20.0%
MSCV-NFAT1 (AP-1 mut)-IRES-GFP
15.9%
CELL NUMBER
GFP

FIG. 6

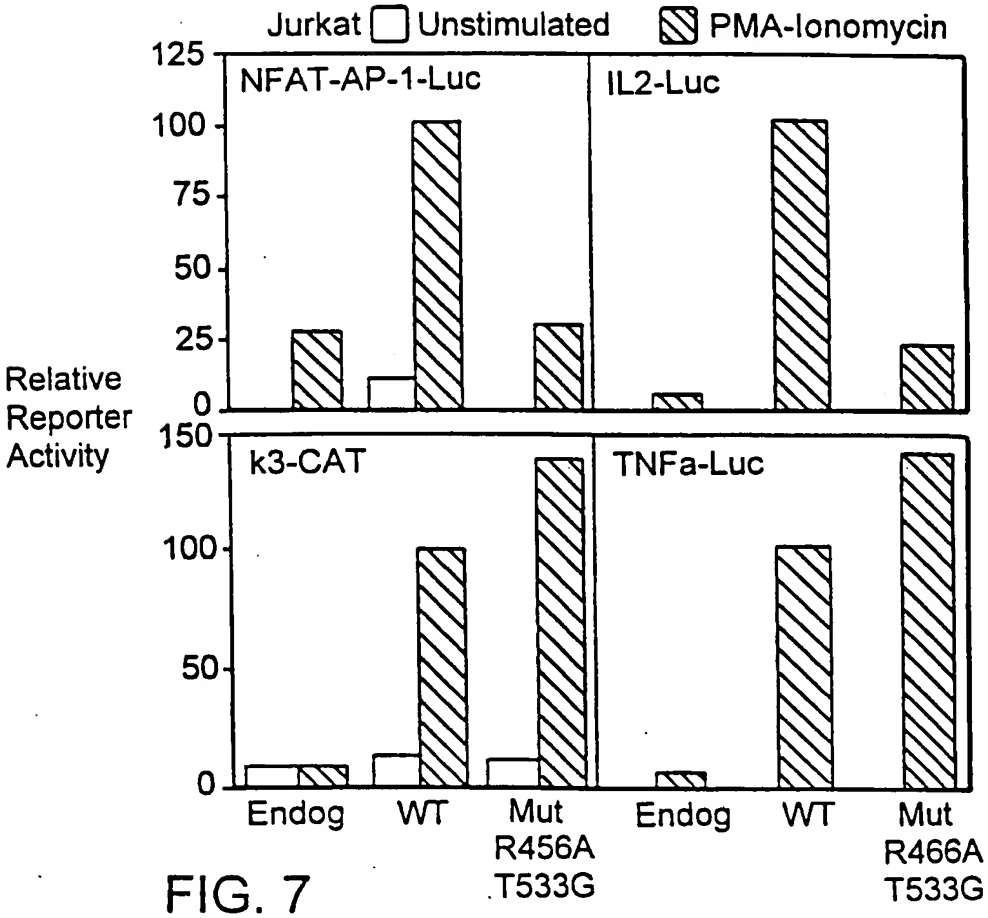
Jurkat
Unstimulated
PMA-Ionomycin
NFAT-AP-1-Luc
IL2-Luc
k3-CAT
TNFa-Luc
Relative Reporter Activity
125
100
75
50
25
0
150
100
50
0
Endog
WT
Mut R456A T533G
Endog
WT
Mut R466A T533G

FIG. 7

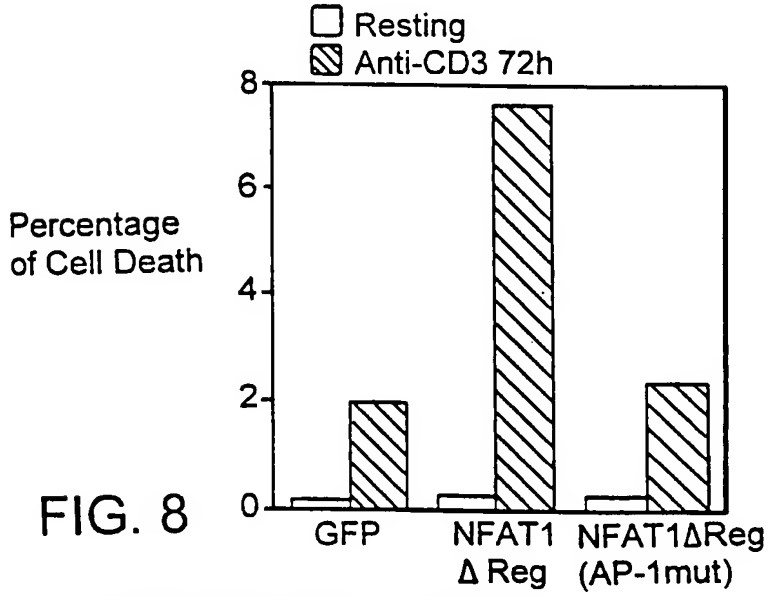
Resting
Anti-CD3 72h
Percentage of Cell Death
8
6
4
2
0
GFP
NFAT1 Δ Reg
NFAT1ΔReg (AP-1mut)

FIG. 8